The role of the Liver X receptors in inflammation: Exploring their contribution to articular pathology.

Volume 1 of 1

by

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Abstract

Background: The Liver X receptors, LXRα and LXRß, belong to the superfamily of nuclear receptor ligand activated transcription factors. LXRs have been well characterised in the context of metabolism through their ability to induce reverse cholesterol transport leading to the excretion of cholesterol from the body. More recently, LXRs have been shown to play a role in inflammation in which they are often ascribed an anti-inflammatory effect. Rheumatoid arthritis (RA) is a chronic auto-immune condition manifest as inflammation of the diarthrodial joints predominantly in the hands and feet. It is now well recognised that RA is not just a local but rather a systemic disease that is associated with several co-morbidities including atherosclerosis. A major focus in the field of rheumatology is now to understand how cardiovascular disease might contribute to the pathogenesis of RA and vice versa and thereby connect metabolism with inflammation. **Hypothesis:** Since LXRs are central to the maintenance of a cholesterol homeostasis and have been shown to regulate inflammation we hypothesised that LXR agonists would be beneficial for the treatment of RA. **Methods & Results:** Treatment of male DBA1 mice with GW3965 or T1317 in the murine model of collagen-induced arthritis dramatically increased the onset and severity of disease. Exacerbation of disease severity was characterised by increased concentrations of multiple serum pro-inflammatory cytokines and chemokines, increased numbers of lymph node derived Th1 and Th17 cells and elevated titres of anti-collagen auto-antibodies. The effect of LXR agonist administration was mediated specifically by LXRs as the severity of disease was not altered in LXR null mice treated with GW3965. Furthermore, activation of LXRs in primary human monocytes potentiated the secretion of multiple proinflammatory cytokines in response to stimulation with LPS. Similarly, the concentration of multiple pro-inflammatory cytokines was also increased in an *in vitro* model of synovitis. **Conclusion:** These studies demonstrate a novel pro-inflammatory role of LXR activation in the context of arthritis. Furthermore, these results suggest that the development of LXR agonists as a therapy for metabolic disorders should be done so with caution.

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3 Acknowledgements

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4 Author’s declaration

The work described in this thesis represents original work which has been generated through my own efforts and does not consist of work forming part of a thesis to be submitted elsewhere. Furthermore, no data has been given to me by anybody else to be submitted as part of my thesis. Where practical support has been provided by others appropriate acknowledgements have been made.

5 Abbreviations

11ßHSD-1	11ß Hydroxysteroid Dehydrogenase Type1
ABCA1	ATP Binding Cassette A1
ACPA	Anti-Citrullinate Protein Antibodies
ACR	American College of Rheumatology
AF-1/2	Activation Function Domain-1/2
AIM	Apoptosis Inhibitor Factor expressed by Macrophages
aP2	acid binding Protein 2
APO	Apolipoprotein
APRIL	A Proliferation Induced Ligand
ARGII	Arginase II
AS	Ankylosing Spondylitis
BLyS	B-lymphocyte stimulator
BMI	Body Mass Index
CFA	Complete Freunds Adjuvant
CIA	Collagen-Induced Arthritis
CRP	C-Reactive Protein
CTLA-4	Cytotoxic T Lymphocyte Associated Antigen 4
CVD	Cardiovascular Disease

CYP7A1	Cholesterol 7α-hydroxylase A1
DBD	DNA Binding Domain
DR	Direct Repeat
EAE	Experimental Autoimmune Encephalomyelitis
ER	Endoplasmic Reticulum
ERR	Estrogen Related Receptor
ESR	Erythrocyte Sedimentation Rate
GLUT 4	Glucose Transporter 4
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GR	Glucocorticoid Receptor
HAT	Histone Acetylase Transferase
IMT	Intima-Media Thickness
IBD	Inflammatory Bowel Disease
IL	Interleukin
FAS	Fatty Acid Synthase
FCS	Foetal calf serum
FXR	Farnesoid X Receptor
ICAM 1	Inter-Cellular Adhesion Molecule 1
LBD	Ligand binding domain

LDLR	Low density lipoprotein receptor
LGK	Liver Glucokinase
LPS	Lipopolysaccharide
LXR	Liver X Receptor
LXRE	LXR response element
MCSF	Macrophage colony stimulating factor
MMP	Matix Metalloproteinase
NPC-1	Niemann-Pick type C1
NR	Nuclear Receptor
Nr1h3/ 2	Nuclear receptor subfamily 1, group h, member 3/ 2
OA	Osteoarthritis
PBMCs	Peripheral Blood Mononuclear Cells
PLTP	Phospholipid Transfer Protein
PPAR	Peroxisome Proliferator-Activated Receptor
PPRE	PPAR response elements
PRRs	Pattern Recognition Receptors
PsA	Psoriatic Arthritis
PXR	Pregnane X Receptor
RA	Rheumatoid Arthritis

RANK	Receptor Activator of NF-kB
RANKL	Receptor Activator of NF-kB Ligand
RF	Rheumatoid Factor
RXR	Retinoid X Receptor
SNP	Single Nucleotide Polymorphism
SREBP-1c	Sterol Receptor Element Binding Protein 1c
StAR	Steroid Acute Regulatory Protein
TBP	TATA Binding Protein
TcKs	Cytokine Activated T cells
TLR4	Toll Like Receptor 4
TNFα	Tumour Necrosis Factorα
TMB	3,3',5,5'-tetramethylbenzidine
UCP-1	Uncoupling Protein 1
UPR	Unfolded Protein Response
VCAM 1	Vascular cell adhesion molecule 1
VEGF	Vascular Endothelial Growth Factor
WAT	White Adipose Tissue

6 General introduction

6.1 Nuclear receptors

6.1.1 Classification of nuclear receptors

Nuclear receptors (NRs) are a super-family of ligand activated transcription factors that represents the largest family of transcription factors in metazoans (1). To date 49 NRs have been identified in mice but only 48 have emerged in humans which lack the Farnesoid X receptor ß (FXRß), the function and consequences of which remain unknown (2, 3). NRs were originally grouped into three broad categories dependent upon their ligands:

- Class I - the steroid family
- Class II - the non-steroid family
- Orphan receptors for which ligands are not known

Profiling the expression of NRs in various murine tissues coincidentally revealed that Class I NRs were expressed in tissues associated with reproduction and central nervous system (CNS) function whereas Class II NRs were expressed in tissues with functions associated with nutrient metabolism and immunity (4). This study also led to the reclassification of the orphan NRs based upon the function of the tissues in which they are expressed. These groups were subsequently further subdivided into Class I A, B and C and Class II A, B and C on the basis of tissue specific expression (Figure 6.1). Grouping of NRs on the basis of tissue specific expression has revealed potential functional characteristics for each NR. Furthermore, grouping of NRs on this basis dictates that LXRα is associated with lipid metabolism whilst LXRß is suggested to predominantly play a role in regulation of the CNS and basal metabolism. Moreover this study has also revealed potential hierarchical networks of NR interactions. The implication of these findings has been supported by other studies. Thus, LXRß is grouped with Rev-erbα and activation of LXRs induces the expression of Rev-erbα which in turn negatively regulates the expression of LXR activation and subsequent target gene expression forming a self regulating feedback loop (5). Similarly, LXRα is found to be co-expressed with the Glucocorticoid Receptor (GR) and the Peroxisome Proliferator Activated Receptor γ (PPARγ). Activation of LXRs has

been shown to both inhibit the synthesis and activation GR ligands but also induce the expression of PPARγ (6-8). So far no interaction of NRs across different groups has yet been reported. These studies demonstrate how the interaction of NRs is defined by spatial separation. However, it is important to take into account another dimension; namely time. Similar studies have also analysed the temporal regulation of NRs and have shown that in adipose tissue and liver 25 NRs are expressed in a rhythmical cycle e.g. Rev-erbα/ß, PPARα/δ/γ and Estrogen related receptor (ERRα/ß/γ) (9). The temporal regulation of NRs in other tissues has not been studied; however, these data suggest that the activities and interactions of NRs may be regulated temporally as well as spatially within the cell. Together these studies support the hypothesis that NRs form hierarchical networks that may serve to regulate the expression, activation and the magnitude of adjacent / parallel NR induced responses. This provides a tightly regulated network of interactions and transcriptional consequences that are now implicated in many aspects of normal physiology and increasingly in pathology.

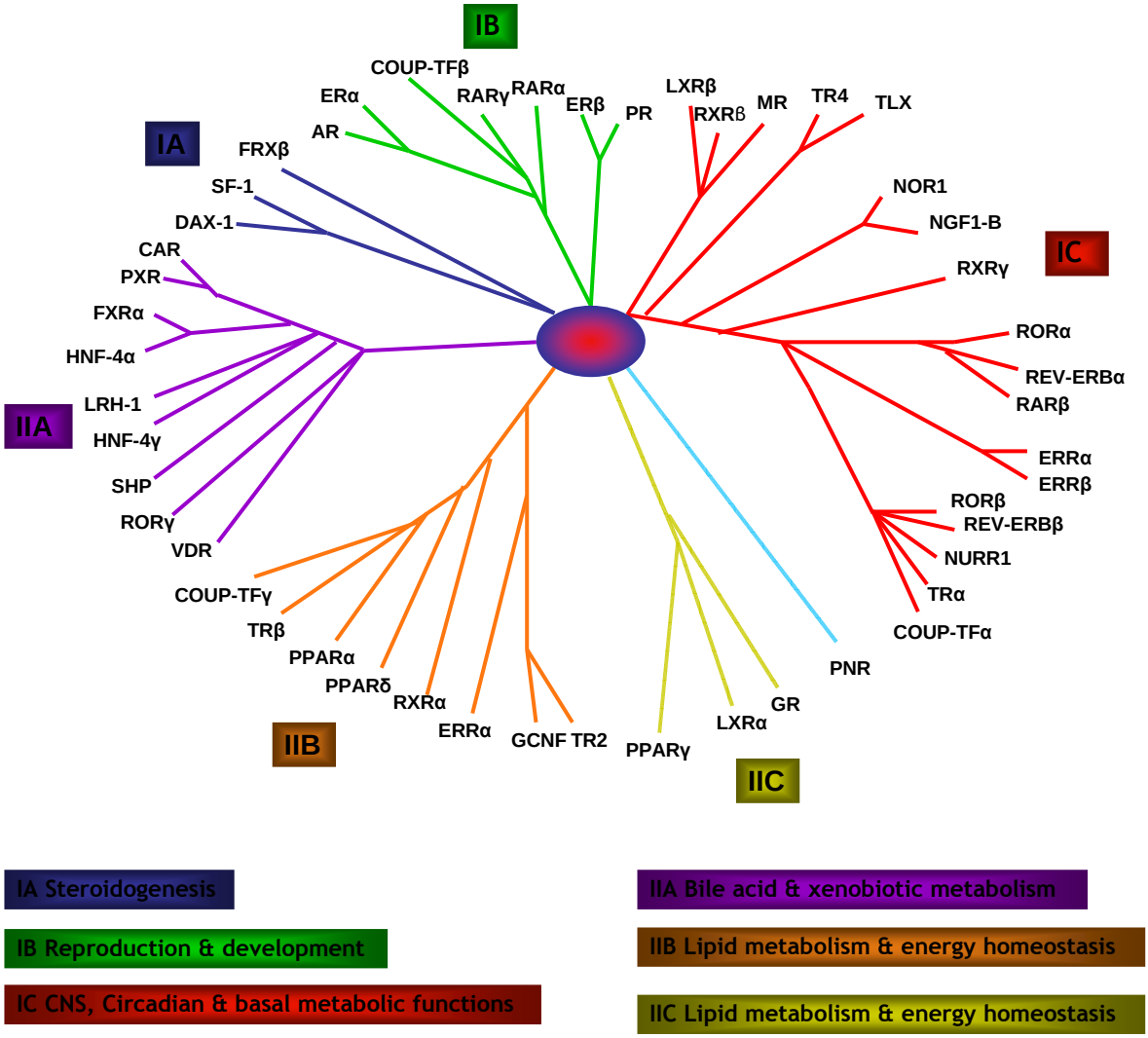


Figure 6.1 Nuclear receptor classification
Classification, grouping and function of nuclear receptors based upon their common anatomical gene expression in murine tissues. This diagram does not represent nuclear receptor phylogeny. Adapted from (4).

6.1.2 Common Nuclear receptor structure

NRs regulate transcription of a variety of genes when activated by the binding of specific lipophilic ligands; these include entities such as vitamins, steroids and lipid molecules. Activation of NRs by ligand binding causes NR localisation to the nucleus where they bind to specific DNA sequences and subsequently recruit transcriptional co-activators thereby inducing transcription of selected target genes. Given this common function all NRs share some common tertiary structural domains which are translated as part a single polypeptide chain (10) (Figure 6.2). Firstly, each NR has a DNA binding domain (DBD) which binds to direct repeats (DR) of a specific hexameric nucleotide sequence separated by between 1-7 non-specific nucleotides. The ligand binding domain (LBD) has multiple functions as well as binding specific ligands. The LBD also contains a

ligand-regulated activation function (AF-2) which is responsible for the binding of co-activators, chromatin remodelling proteins such as histone acetyltransferases (HATs), required to “open up” chromatin for the initiation of transcription. Secondly, the ligand binding domain (LBD) has been implicated in the dimerisation of NRs which is required for stringent binding to response elements within the promoters of target genes (11). Most NRs also have a ligand regulated transcription activation function domain (AF-1) towards the N terminus of the DBD. This has been shown to exhibit broad diversity across NRs and as such has been proposed to be responsible for distinct effects of similar NRs through the binding of different transcriptional co-activators. Additionally, the AF-1 domain can synergise with AF-2 to mediate the magnitude of a transcriptional response by some NRs (12).

Figure 6.2 Common nuclear receptor structure
All nuclear receptors share some common structural components; a DNA binding domain (DBD), a ligand binding domain (LBD), which contains a ligand regulated transcription activation function domain (AF-2) and most nuclear receptors and a transcription activation function domain (AF-1) which can synergise with AF-2 to bind transcriptional co-activators (Adapted from (10)).

6.2 Liver X receptors: Structure & Function

6.2.1 Identification of Liver X Receptors

The Liver X Receptors (LXRs) were first identified as members of the nuclear receptor superfamily of transcription factors in 1994 (13). Since their initial discovery two members have been identified, LXRα and LXRß: although they are often referred to as “isoforms” LXRα and LXRß are encoded by distinct genes on separate chromosomes (14). At the time of their discovery the endogenous ligands for LXRα and LXRß were unknown and were therefore described first as orphan receptors.

6.2.1.1 LXRα

LXRα was identified in 1995, one year after LXRß, and because of its high level of expression and subsequent purification from the liver the name “Liver X Receptor” was coined with the “X” referring to the lack of an identified ligand (14). As well as having a high level of expression in the liver, northern blot analysis also revealed a high level of expression in kidney, spleen, intestine and the adrenals, subsequently the expression of LXRα has also been demonstrated in adipocytes, macrophages, T cells, B cells, neutrophils and dendritic cells in both mice and humans (15-19). The anatomical expression profile dictates that LXRα is a Class II C NR, grouped with PPARγ and GR, in tissues which predominantly regulate lipid and energy homeostasis (4) (Figure 6.1). LXRα is encoded by the gene NR1h3 which is located on chromosome 11 in humans and chromosome 2 in mice. The murine LXRα gene spans approximately 11 Kb of DNA and consists of 10 exons which encode a transcript of approximately 1137 bp in length (20).

6.2.1.2 LXRβ

LXRß was firstly described as “Ubiquitous Receptor” (UR) due to its ubiquitous expression in a widespread number of analysed rat tissues detected by Northern blot analysis; other synonyms included OR-1, Rip-15 and NER (13, 21-23). Using a probe homologous to the DBD of other known rat NRs a 1.9 Kb cDNA sequence was identified which did not code for any previously recognised NRs but was subsequently later identified as human LXRß. The purified rat cDNA was subsequently cloned, sequenced and used to identify a cDNA sequence from human prostate cancer cells which had 90% homology. UR was later renamed LXRß based upon the high level of similarity with LXRα, which share 77% amino acid sequence similarity within both the LBD and DBD (14) and has been positioned in class I C, grouped with RXRß (Figure 6.1). Murine LXRß is encoded by the gene, Nr1H2, located on chromosome 19q13.3. Nr1H2 consists of 9 exons from which a cDNA sequence of 1652 bp has been identified (20).

6.2.1.3 The Liver X Receptors are evolutionary conserved

Both LXRα and LXRß have been conserved throughout evolution and as such human LXR orthologs have been identified in worms (*caenorhabditis elegans)*, flies (*drosophila melongaster)*, fish (*fugu ruripes)*, frogs (*xenopus laevis)* and mice (*mus musculus*). Murine LXRs are evolutionary closely linked to humans (14, 24-26). Indeed there is a high degree of amino acid sequence homology of the LBD (98% and 94%) and DBD (95% and 90%) for LXRα and LXRß respectively between humans and rodents (13, 14). Furthermore, recent studies have demonstrated robust activation of LXRα and LXRß by both synthetic and endogenous ligands in many species throughout evolutionary history (24). These studies would suggest that the LXRs have a long evolutionary history and that their ability to be activated and bind target sequences has been conserved. In addition these data provide support for the use of rodents as a good *in vivo* model system to elucidate the biology of LXRs.

6.2.2 Liver X receptor ligands

The activation of LXRs is therefore regulated post-translationally predominantly by the binding of endogenous ligands. In screening efforts to identify a potential ligand, retinoids were found to activate the expression of a luciferase reporter which was ligated in turn to a promoter bound by LXRα (14). However, retinoids had previously been shown to specifically activate the Retinoid X Receptors (RXRs) and further studies revealed that activation of LXR induced transcription was mediated through heterodimerisation with RXR (27). Cholesterol loading of macrophages has been shown to induce LXR activation thereby implicating LXRs as sensors of cholesterol homeostasis. However, the induction of LXR activation by cholesterol has since been shown to be mediated by oxidised cholesterol derivatives; oxysterols (28). Oxysterols can be formed by direct ‘attack’ from reactive oxygen species or may be absorbed pre-formed in the diet. However, the primary pathway of oxysterol formation is by the enzymatic oxidation of cholesterol during cholesterol degradation and synthesis by members of the cytochrome family of enzymes. Several species of oxysterols were first shown to induce LXRα activation *in vitro*; particularly 22(R) hydroxycholesterol, 24(S) hydroxycholesterol, 20(S) hydroxycholesterol and 24(S),25 epoxycholesterol (Figure 6.3) (28, 29). Furthermore, it is now known that LXRß is also activated by

similar oxysterol ligands reflecting the high degree of amino acid sequence conservation of the LBD between LXRα and LXRß (14, 30). However, not all oxysterols function as LXR agonists as 22(S) hydroxycholesterol has recently been shown to have inhibitory effects upon LXR activation and subsequent downstream signalling both *in vitro* and *in vivo* (31, 32).

Subsequently, several synthetic LXR agonists have been designed which are widely used for the investigation of LXR biology (Figure 6.3). Similar to the oxysterol ligands these synthetic agonists are active in many species including mice and humans (24). Firstly, Tularik (Amgen) developed T0901317 (T1317) and demonstrated that treatment of HEK293 cells with this agent potently induced the activation of both LXRα and LXRß, although the magnitude of LXRα activation was greater than that of LXRß; 35 fold relative to 15 fold respectively (33). GlaxoSmithKline later developed a non-steroidal agonist (GW3965) which exerted similar levels of potency with respect to the activation of LXRα compared to LXRß and was shown to successfully induce the expression of the LXR target gene ABCA1 *in vivo* (34). More recently several other companies have developed LXRα/ß dual agonists and demonstrated similar effects upon the induction of LXR target genes however their effects remain to be confirmed by other investigators (35, 36). Furthermore, elucidation of the individual role of either LXRα or LXRß has until recently been restricted to the use of knockout animals as agonists that distinguish between the two did not exist. However, there are now reports of agonists that specifically activate LXRα or LXRß and will undoubtedly prove useful in separating the individual roles of LXR activation (37). In addition a synthetic LXRα/ß dual antagonist has been described although its specificity with respect to its effect upon other NRs remains unknown (38).

In addition to sensing alterations in cholesterol concentrations LXRs have also been implicated in the regulation of carbohydrate metabolism. Recently glucose has been shown to activate both LXRα and LXRß *in vitro* where it was shown to induce the expression of a luciferase reporter at levels comparable to synthetic LXR agonists (39). Moreover, this was shown to be a direct effect as glucose was shown to displace the high affinity LXR ligand T1317 from an LXRE containing promoter, recruit transcriptional co-activators and induce transcription of multiple LXR target genes.

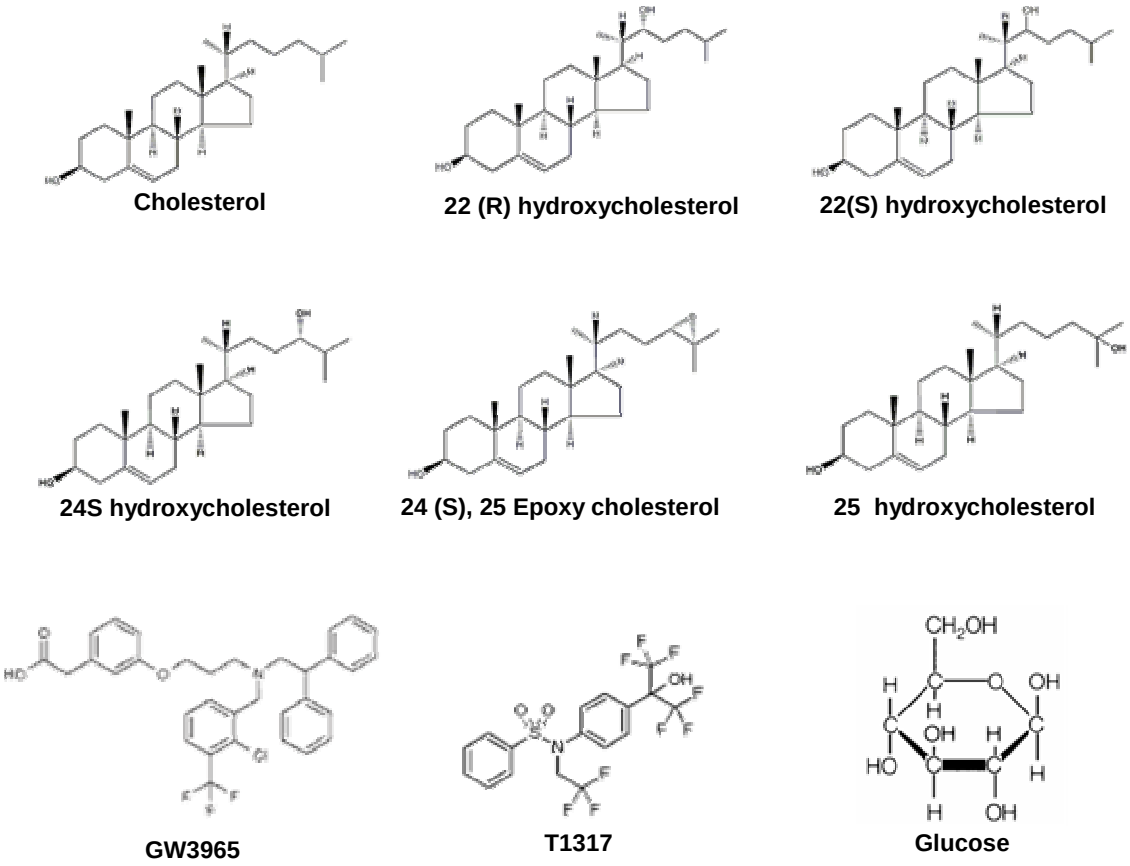


Figure 6.3 Endogenous and synthetic LXR ligands
The structure of the oxysterols which have been identified as endogenous LXR ligands and the most commonly used synthetic LXR agonists GW3965 and T1317. Glucose has recently been identified as a direct activator of LXRs.

It is therefore evident that there are a multitude of potential LXR ligands all of which are intermediates of metabolic processes; in particular they subserve either lipid or carbohydrate metabolism. How these different endogenous ligands interact and impact the activation of LXRs and down stream target genes in physiology is of great interest but remains poorly understood reflecting the lack of specificity noted above. However, many studies utilising the synthetic LXR agonists have identified a large number LXR target genes and have therefore expanded our understanding of LXR biology.

6.2.3 Regulation of LXR expression

LXRα and LXRß are both constitutively expressed across a broad distribution of tissues. However, it is possible to modulate the level of LXRα or LXRß expression through tissue specific exogenous and endogenous signalling pathways which alters synthesis of LXR transcripts. Changes in LXR expression may ultimately

lead to altered LXR induced responses. However, only a few studies have focussed upon the factors that regulate the expression of LXRs. As such this is an area of considerable interest in terms of understanding their inducible biology.

After the identification of the genes that encode the LXRs, promoter analysis revealed several potential transcription factor binding sites for SP1 (20). SP1 was known to bind and regulate the expression of multiple house keeping genes and it was proposed that SP1 may be responsible for regulating the basal expression of LXRα and LXRß. However, binding of SP1 to an LXR promoter and regulation of LXR expression by SP1 has never been demonstrated, it therefore remains unknown how the basal level of the LXRs' expression is maintained.

At the same time potential NF-κB and AP-1 binding sites were also identified in the promoter of LXRß which have since been confirmed by other studies. Since these signal transducers are implicated primarily in leukocyte activation, this is compatible with an additional role of LXRs in immunity. Treatment of human hepatic and kidney cell lines with TNFα *in vitro* inhibits the expression of both LXRα and LXRß (40-42). However, contradictory results have been obtained in two further studies where the expression of LXRs has been shown to be increased in rabbit adipocytes treated with TNFα (43, 44). TNFα also clearly activated NF-κB in these latter studies which suggest that TNFα signalling, mediated by interactions of NF-κB with the LXR promoter, may lead to different outcomes upon LXR expression in distinct cell types. Microarray analysis has also demonstrated that the expression of LXRα was increased upon the differentiation of human monocytes with granulocyte/ macrophage-colony stimulating factor (GM-CSF) to macrophages (45).

Activation of LXRs in human macrophages by cholesterol loading and treatment with synthetic or endogenous ligands has been shown to up-regulate the expression of LXRα (46-48). This capacity was lost upon mutation of LXR binding sites within the LXRα promoter suggesting that the expression of LXRα is positively auto-regulated. However, this auto-regulatory loop is (i) specific to the induction of LXRα expression and not LXRß, and (ii) is likely to be cell type specific since it was not observed in human adipocyte and hepatic cell lines. Furthermore, the induction of the LXR target gene ABCA1 expression was greater in human macrophages compared to adipocytes or hepatocytes suggesting that

this auto-regulatory loop serves to further enhance the induction of LXRα target genes specifically in macrophages. In contrast to macrophages, adipocytes and hepatocytes apparently lack LXR associated signalling pathways responsible for the increased expression of LXRs in human macrophages. Moreover, all studies concur in showing that the ability of LXRα to auto-regulate its expression is specific to macrophages of a human origin as induction of LXRα expression in mouse macrophages was not observed upon treatment with LXR agonists. Further analysis revealed that the murine LXRα promoter was found to lack the 5' region containing the LXRE explaining the lack of LXRα auto-regulation in mouse macrophages. This is indicative of a species difference in the mechanism regulating the expression of LXRα. These studies have important implications for LXR biology and add another level of complexity to the regulation of LXR transcription. However, caution should be applied when comparing the regulation of LXRs and consequent transcription of designated target genes between mice and humans. This is also relevant in considering interpretation of murine *in vivo* model studies and human analyses as will become important later in my studies.

Previous investigations studying the regulation of LXR expression have shown that PPARγ ligands were able to upregulate the expression of LXRα in human and mouse macrophages and consequently LXRα target genes (47, 49). Furthermore, sequence analysis identified PPAR response elements (PPRE) within both the human and mouse LXRα promoters'. Additionally, treatment with LXR and PPARγ ligands drive the expression of LXRα and the effect is synergistic when the ligands are added in combination (47). This demonstrates once again how multiple signalling pathways may interact to regulate the transcription of LXRs.

6.2.4 LXR splice variants

Ongoing attempts to decipher the mechanisms by which LXR expression is regulated have identified two additional novel transcripts encoded by the human LXRα gene, NR1H3 (50). Similarly, alternative transcript start sites have also been identified in the NR1H3 gene in mice (20). LXRα sequence analyses have identified these transcripts as alternative splice variants of LXRα and have been named LXRα2 and LXRα3; where LXRα1 (referred to throughout as LXRα) is the

originally described full length transcript. LXRα3 is transcribed from the same promoter as LXRα1, however exclusion of exon 6 caused an in frame deletion of 50 amino acids from the LBD. The transcription of LXRα2 is regulated by an alternative promoter, 5' of the originally identified promoter sequence, and is lacking 45 amino acids in the N terminus AF-1 domain in LXRα1. These structural differences are illustrated in Figure 6.4. The sub-cellular localisation of the three LXRα isoforms was identical; i.e. predominantly nuclear. However, HEK-293 cells transfected with LXRα2 or LXRα3 expression vectors and treated with the LXR agonist T1317 revealed that the induction of ABCA1 transcripts was diminished. These data are suggestive of a decreased capacity of LXRα2 and LXRα3 to be activated by known LXR ligands which may be mediated through an altered ability to recruit transcriptional co-activators or bind agonist respectively. Importantly the transcription of alternative LXRα splice forms may provide a regulatory mechanism for the induction of LXR target genes.

LXRα1 is known to be expressed in a wide variety of tissues; however in comparison LXRα2 and LXRα3 are expressed at very low levels except in the testes where LXRα2 is highly expressed. Additionally, LXRα2 and LXRα3 were expressed at a level comparable to that of LXRα1 in several tumour cell lines including Thp-1 cells; notably the expression in primary human monocytes/ macrophages was not assessed. Since, the isoforms are expressed at a higher level in tumour cell lines this may suggest that LXRα2 and LXRα3 might have pathophysiological roles in disease; although this remains to be explored. However, the identification of similar LXRα transcripts in the mouse has not been reported to date. Additionally, a pseudogene for murine LXRβ (ΨLXRβ) has been identified which contains multiple point mutations and deletions in comparison to LXRβ cDNA (20). The implications for the (potential) physiological role of these LXRα isoforms is not clear however, it is possible that their expression may be induced by specific metabolic or inflammatory stimuli. Further studies are required to elucidate their endogenous roles.

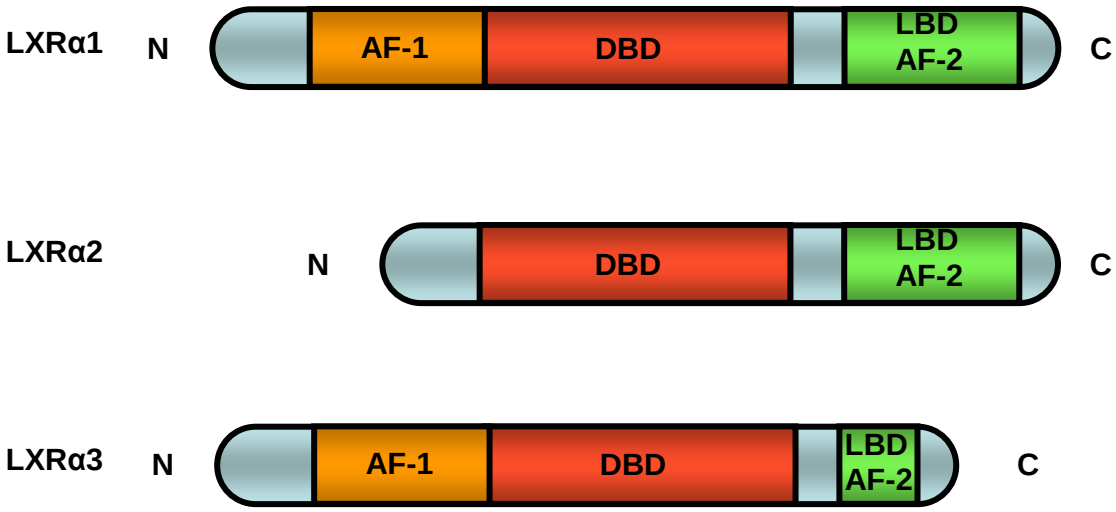


Figure 6.4 Alternative splice forms of LXRα.
A schematic of the protein polypeptides of the three human LXRα splice variants. LXRα1 is encoded by the full length transcript to form the AF-1 domain, DNA binding domain (DBD) and the ligand binding domain (LBD). LXRα2 is truncated at the N terminus and is formed by use of an alternative promoter and removal of an exon encoding the AF-1 domain. LXRα3 is truncated at the C terminus resulting in partial deletion of the LBD.

6.2.5 Regulation of Liver X Receptors activation & transcriptional activity

The expression and activation of LXRs is tightly regulated - within the nucleus LXRs are in complex with the nuclear receptor Retinoid X Receptor (RXR) forming LXRα/RXR or LXRβ/RXR heterodimers (14). In the absence of ligand the LXR/ RXR heterodimer complex is present in an inert conformation and is bound by transcriptional co-repressors rendering it transcriptionally inactive. Recently the corepressors nuclear receptor corepressor (NCoR) and silencing mediator of retinoic acid and thryroid receptor (SMRT) have been implicated in the regulation of LXR target genes (51). They function by recruiting additional transcriptional repressor molecules such as histone deacetylases (HDACs) forming large corepressor complexes that promote a ‘closed’ chromatin conformation. However, binding of an agonistic ligand to the LXR LBD induces a conformational change of the LXR/RXR heterodimer releasing the corepressor complex and allowing binding of coactivator complexes and transcription of LXR target genes (52). It has been suggested that the extent of LXR activation may be mediated by differential cofactor recruitment; indeed partial agonism of LXR has been shown to be due to the recruitment of the corepressor NCoR (53).

LXRs are predominantly localised within the nucleus regardless whether or not they are bound by ligand (54). However, there is a growing body of evidence demonstrating that LXRα, but not LXRß, can translocate between the cytoplasm and the nucleus even when bound by agonist. This may impose additional levels of regulation upon the transcriptional activity of LXRα (55). Retention of LXRα within the nucleus has been shown to be in part regulated by TNFα induced phosphorylation within the AF2 region of LXRα (56-58). Additionally, a nuclear localisation sequence has been identified on both LXRα and RXR which are essential for nuclear import, although RXR mediated translocation of LXR can not induce transcription of LXR target genes (59). The activation of LXRs is summarised in Figure 6.5.

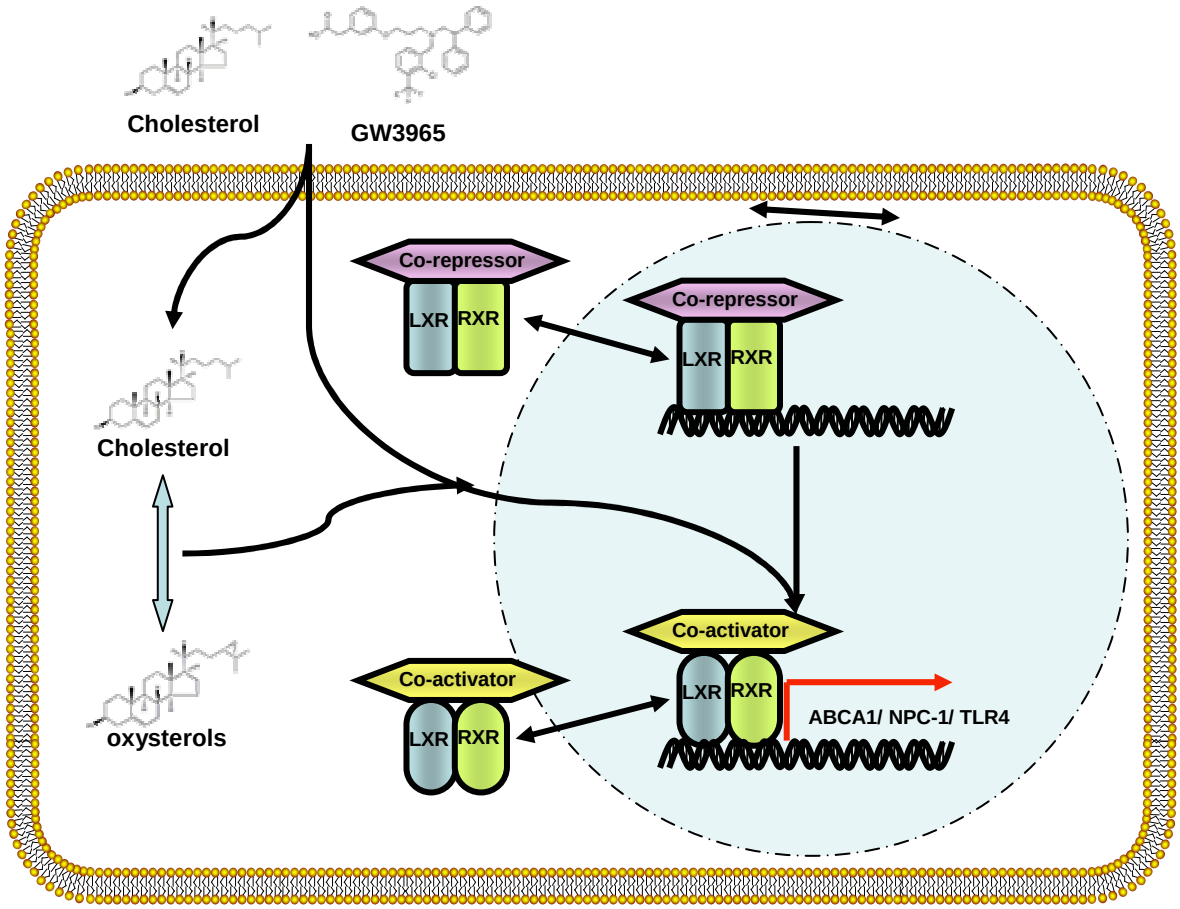


Figure 6.5 Regulation of LXR transcriptional activation
Oxygenated forms of cholesterol (oxysterols) or synthetic LXR agonists bind to the ligand binding domain in LXRα or LXRβ which are in partnership with RXR. Ligand binding induces the conformational change of the LXR/RXR complex inducing the release of transcriptional co-repressors and allowing the binding of transcriptional co-activators thereby switching on target gene transcription. LXRα, but not LXRβ, can be translocated between the cytoplasm and the nucleus and vice versa even in the presence of agonist.

Upon activation of LXRs within the nucleus they can induce transcription by binding to LXR response elements (LXRE) within the promoter region of designated target genes. LXREs are formed from two hexameric direct repeats (DR) separated by 4 nucleotides as such they are designated DR4 type response elements (60). Many genes have now been identified as being directly regulated by LXRS by the presence of LXRE within their promoters and in the context of reporter based assays. However, although LXRs have been implicated as having an immuno-modulatory role most of the LXR target genes that have been identified have been ascribed functions regulating lipid metabolism and transport e.g. ABCA1, NPC-1 and PLTP. To date the only LXR target genes that have been identified with a direct role in inflammation are those encoding CCL24, human TLR4 and human MPO (61, 62). A full list of known LXR target genes along with a brief description of their assigned functions is shown in Table 6.1.

Physiological role	Target gene	Assigned function	Ref
Adiposity and glucose metabolism	aP2 ↑	Specifically expressed in adipocytes. Activated by binding of fatty acids and regulates adipocyte differentiation.	(63)
	GLUT4 ↑	Widely expressed glucose transporter of which the expression in epididymal white adipose tissue is regulated by LXRα.	(64)
	LGK ↑	Predominantly expressed in the liver, pancreatic ß cells and the brain where it phosphorylates glucose to glucose-6-phosphate for entry into glycolysis or to be stored as glycogen. LGK expression is also regulated by the LXR target gene SREBP-1c thereby amplifying the level of expression.	(65)
	PPARγ ↑	Predominantly expressed in adipocytes and is the master regulator of adipocyte differentiation. PPARγ activation ameliorates type II diabetes, cardiovascular disease and has been shown to exert anti-inflammatory effects.	(6, 66)
	PPARα ↑	PPARα is associated with anti-inflammatory effects and regulates uptake of triglycerides and glucose in cardio myocytes.	(67, 68)

	UCP-1 ↓	Inhibition mediated by LXRα. UCP-1 regulates thermogenesis in brown adipose tissue	(69)
Lipid transport and metabolism	ABCA1 ↑ ABCG1 ↑	Promote efflux of cholesterol from macrophage foam cells onto apoA-I and HDL particles and therefore promotes reverse cholesterol transport. Mutations in ABCA1 are associated with Tangier disease.	(49, 70, 71)
	AIM ↑	Member of the scavenger receptor cysteine-rich superfamily which secreted by and protects macrophages from apoptosis. Induction of AIM is regulated by LXRα, but deletion of AIM protects against atherosclerosis. (Alias SPα).	(72)
	Arg II ↑	Catalyses the conversion of L-Arginine to ornithine and urea. Arginine is required for nitric oxide synthesis. Arginase II inhibits the production of nitric oxide by inflammatory cells.	(73, 74)
	ApoD ↑	Apolipoprotein which transports cholesterol as part of HDL particles from the periphery to the liver for excretion from the body.	(75)
	ApoE ↑	Transports cholesterol as part of LDL and VLDL particles from the periphery for degradation and excretion as bile through binding to LDL receptors in the liver. ApoE4 alleles are associated with Alzheimer's disease.	(70, 76)

	ApoA-V ↑	Synthesised in the liver and secreted into the plasma where it is associated with VLDL and HDL and regulates the concentration of triglycerides. SNPs in the human population correlate with increase triglyceride levels.	(77, 78)
	CD36 ↑	Scavenger receptor mediated in the cellular uptake of free fatty acids in the liver and oxLDL on macrophages. Direct induction of CD36 by LXRs is unique to the liver, although CD36 is expressed on a variety of cell types.	(79)
	CYP7A1 ↑	Regulates the rate limiting step of bile acid synthesis which can in turn repress CYP7A1 through activation of FXR.	(80)
	FAS ↑	Synthesis of fatty acids (conversion of melanyl CoA to palmitate) and subsequent synthesis and elevation of serum and hepatic triglycerides.	(33, 81, 82)
	LDLR ↑	Mediates the endocytic uptake of LDL cholesterol in the liver which has been transported by lipoproteins (predominantly HDL) from the periphery. Induction of LDLR can be induced by LXRα.	(83)
	NPC-1 ↑	Regulates trafficking of cholesterol from the cholesterol endocytic pathway to the plasma membrane and endoplasmic reticulum. Activation of NPC-1 has been implicated in the amelioration of atherosclerotic plaques in $ApoE^{-/-}$ mice through	(84-86)

		activation of LXRs. Mutation in NPC-1 is causes Niemann-Pick C disease resulting in early death.	
	PLTP ↑	Transfer of phospholipids from VLDL particles to HDL particles during reverse cholesterol transport. Also has been implicated in the neutralisation and clearance of lipopolysaccharide by transfer from HDL to LDL.	(87-89)
	RENIN ↑	A protease expressed in the kidney which converts angiotensinogen to angiotensin I which induces the secretion of aldosterone from the adrenal gland to promote salt absorption.	(90)
	SREBP-1C ↑	Transcription factor that regulates the expression of glycolytic and lipogenic genes e.g. FAS, LDL receptor and glucokinase.	(6, 91, 92)
Steroidogenesis	11β-HSD-1 ↓	Is expressed in liver, adipose and macrophages and regulates the activation of inert cortisone into biologically active cortisol. Modulation of 11β-HSD1 expression therefore impacts upon GC signalling through the GR.	(7)
	GR ↓	A nuclear receptor activated by the binding of corticosteroids or synthetic steroids.	(93)

	StAR ↓	Rate limiting enzyme involved in the synthesis of *de novo* glucocorticoids in the adrenal glands from cholesterol.	(94)
Inflammation	CCL24 ↑	Chemokine secreted by monocytes and T cells that interact with the chemokine receptor CCR3 to induce chemotaxis of eosinophils and basophils. Expression of CCL24 can be induced phosphorylation of LXRα.	(56)
	TLR4 ↑	Binds LPS and activates NF-κB inducing the transcription, translation and secretion of a variety of pro-inflammatory cytokines and chemokines. LXR only regulates TLR4 expression in humans.	(5, 61)
	MPO ↓	LXR down-regulates the expression of myeloperoxidase only in human but not mouse macrophages and neutrophils.	(62)

Table 6.1 LXR target genes
A description of genes that have been shown to be directly transcriptionally regulated by the activation of LXRs in mice and humans and a brief description the assigned function in the context of LXR activation. ↑ or ↓ represents increased or decreased expression of target gene respectively. Adipocyte fatty acid binding protein 2 (aP2), glucose transporter (GLUT4), liver glucokinase (LGK), peroxisome proliferator activated receptor (PPAR), Uncoupling Protein 1 (UCP-1), ATP binding cassette A1/G1 (ABC A1/G1), apoptosis inhibitor factor expressed by macrophages (AIM/ SPα), arginase II (Arg II), apolipoprotein D (ApoD), apolipoprotein E (ApoE), cholesterol 7 alpha-hydroxylase A1(CYP7A1), fatty acid synthase (FAS), low density lipoprotein Receptor (LDLR), Niemann-Pick C1 (NPC-1), phospholipid transfer protein (PLTP), sterol regulatory element binding protein 1c (SREBP-1c), 11β hydroxysteroid dehydrogenase type 1 (11β HSD-1), glucocorticoid receptor (GR), steroidogenic acute regulatory protein (StAR), CCL24 (Eotaxin), toll like receptor 4 (TLR4) and myeloperoxidase (MPO).

In addition to the well recognised role as a transcription factor LXRß has more recently been shown to exert regulatory effects upon LXR target gene products post translationally (95). In a low cholesterol environment LXRß was shown to associate with ABCA1 at the plasma membrane thereby rendering ABCA1 inactive. However, upon cholesterol accumulation LXRß dissociated from ABCA1 promoting ABCA1 mediated reverse cholesterol transport. This is the first example where LXRs have been shown to regulate both the transcription and activity of a target gene product and highlights a novel way in which the regulatory aspects of LXRs might be considered in complex inflammatory systems.

6.2.6 Liver X Receptors in physiology & disease

6.2.6.1 Atherosclerosis

Several studies using synthetic LXR agonists have demonstrated beneficial effects of LXR activation upon atherosclerosis in apolipoprotein E knockout (ApoE- $^{-/-}$) and Low Density Lipoprotein Receptor null (LDLR$^{-/-}$) mice fed a high fat diet. Systemic administration of either of two LXR agonists, T1317 and GW3965, can inhibit the development of, and promote the regression of established atherosclerotic lesions (96, 97). These observations were associated with a beneficial lipid profile; reduced total and VLDL cholesterol but increased HDL. Amelioration of atherosclerotic plaques by LXR agonists has been shown to be mediated primarily through the efflux of cholesterol from macrophage foam cells through the increased expression of the cholesterol transporters ABCA1 and ABCG1. More recently many more effects of LXR activation have now been identified and implicated in the reversal of atherosclerotic plaque development. LXRs have been shown to up-regulate the expression of the Niemann- Pick protein C-1 (NPC-1) (85). NPC-1 regulates intra-cellular cholesterol trafficking and deletion of NPC1 promotes atherosclerotic lesion formation (98). NPC-1 is also involved in the generation of oxysterols which may activate LXRs; increased expression of NPC-1 may therefore provide a positive feedback loop upon the activation of LXRs and subsequently up-regulate the expression LXR target genes involved in reverse cholesterol transport e.g. ABCA1 and NPC-1. Activation of

LXRs has also been shown to inhibit the uptake of cholesterol loaded LDL by macrophages thereby preventing foam cell formation (99). More recent studies in primates have demonstrated that administration of an LXR agonist reduced the concentration of total and LDL cholesterol in a dose dependent manner whilst increasing the expression of ABCA1 (100).

Such studies in rodents and primates have provided support that administration of LXR agonists may be beneficial for the treatment of atherosclerosis. However, it is evident that the dual activation of LXRα and LXRß in rodents, as with T1317 or GW3965, causes the undesirable effects of hepatic steatosis and hypertriglyceridaemia which may lead to liver cirrhosis. These lipogenic effects are mediated through the increased induction of fatty acid synthase (FAS), CD36 and sterol regulatory element binding protein 1c (SREBP-1c) expression. These genes have been shown to be predominantly regulated by LXRα. As such the activation of LXRß in LXRα$^{-/-}$, ApoE$^{-/-}$ double KO mice has been shown to reduce the size of atherosclerotic plaques but did not induce hepatic steatosis or hypertriglyceridaemia (101, 102). These studies therefore suggest that the development of agonists that specifically activate LXRß may be more beneficial for the treatment of atherosclerosis in humans. Given the high level of homology in the LBD between LXRα and LXRß this may prove to be problematic although there are now reports emerging demonstrating the development of compounds with a degree of LXRß selectivity (103). The specificity of such compounds remains to be confirmed by additional groups and there efficacy is yet to be assessed in models of atherosclerosis.

Atherosclerotic plaques are associated with chronic low grade inflammation and as LXRs have been ascribed a recent novel role in inflammation LXR agonists have also been suggested to inhibit inflammation associated with CVD. Indeed, serum analysis by ELISA and hepatic microarray gene analysis demonstrated a decrease in the pro-inflammatory cytokines IL-1 and IL-6 in ApoE$^{-/-}$ mice treated with T1317 (104). However, although the investigators reported a predominantly anti-inflammatory effect the administration of T1317 was associated with an increase in TNFα and MMP9. Additionally, oxysterols, which activate LXRs, have been shown to enhance the expression of the adhesion molecules, inter-cellular adhesion molecule 1 (ICAM 1) and vascular cell adhesion molecule 1 (VCAM1) (105). ICAM1 and VCAM1 are expressed on the surface of endothelial cells and

direct leukocyte migration into tissue and blood vessels during atherosclerotic lesion development. The effect of LXR activation upon the inflammatory component of atherosclerosis is therefore not clear. Furthermore, the development of LXRß agonists for the treatment of atherosclerosis should be done so with caution as the effect of LXRß agonism upon inflammation has not been studied and the effects unknown.

6.2.6.2 Obesity

Obesity was originally regarded as simply storage of excess fats within white adipose tissue (WAT); however, it is now evident that obesity is associated with low grade chronic inflammation. Adipocytes in WAT are situated in close proximity to macrophages, dendritic cells and lymphocytes (106-110) in which both LXRα and LXRß are highly expressed. Similarly, the nuclear receptor PPARγ is highly expressed in adipocytes where it acts as the master regulator of adipocyte differentiation and regulates the expression of LXRs during adipocyte development (111). The role of LXRs in adipogenesis and adipocyte lipid accumulation has been investigated. Lipid accumulation in LXRα/ß double knockout mice is reduced when fed a high fat diet over a prolonged period of 12 months in which the size of adipocytes and WAT is reduced (112). Similarly, LXR agonism during adipocyte differentiation has been shown to promote a greater accumulation of lipids (111). This effect was shown to be LXRß specific as $LXR\alpha^{-/-}$ mice do not exhibit the same phenotypic changes in the reduction of adipocyte and WAT size. It has now been determined that LXRs do not affect adipogenesis, but do regulate lipid metabolism within adipocytes (112-115). Reduced lipid accumulation in adipocytes deficient in LXRß has been proposed to be regulated through the Uncoupling Protein 1 (UCP-1). UCP-1 regulates mitochondrial respiration and increased expression of UCP-1, as seen in $LXR\beta^{-/-}$ adipocytes, leads to the oxidation of lipids without the production of ATP. Similar to observations seen in rodent models the expression of LXRα was found to be significantly increased in WAT from obese women. A role of LXRs in obesity has further been confirmed through human genetic analysis. Recently several single nucleotide polymorphisms (SNPs) were found in Nr1h3 and Nr1h2 which were shown to associate with increased and decreased Body Mass Index (BMI) (116). This is suggestive of a genetic predisposition to either a protective or predictive

phenotype for the onset of obesity which might be regulated by an altered functional activity of LXRs.

Obesity is often associated with type II diabetes. As well as reduced adiposity LXRß$^{-/-}$ mice are also glucose intolerant and exhibit impaired glucose induced insulin secretion from pancreatic islet cells (112). Furthermore, activation of LXRs has been shown to improve glucose tolerance through the increased expression of an insulin sensitive glucose transporter (GLUT4) and suppression of gluconeogenesis (114). Since macrophages are involved in both inflammation and metabolism a role for them in LXR driven glucose tolerance was investigated. However, transfer of LXRα/ß double knockout bone marrow into wild-type recipients was shown to have no effect upon net body weight gain and glucose tolerance (113), although the effect specifically upon adipose associated inflammation was not assessed.

6.2.6.3 Autoimmunity and inflammation

LXRs have a well defined role in tissues which exhibit metabolic functions and as regulators of cholesterol homeostasis. More recently LXRs have been implicated as having a modulatory effect upon inflammation in which they have generally been ascribed an anti-inflammatory function. However, the role of LXRs in inflammation is controversial and the mechanism by which they modulate inflammatory processes remains largely unknown.

LXR agonism was first suggested to exert potent anti-inflammatory effects exemplified in the suppression of pro-inflammatory cytokine secretion from lipopolysaccharide (LPS) stimulated murine macrophages treated by GW3965, T1317 or endogenous agonistic LXR ligands (117). Inhibition of the pro-inflammatory mediator Cox-2 by this route was shown to be NF-kB dependent. LXR activation effectively inhibited the expression of a Cox-2 luciferase reporter which was lost upon mutation of the NF-kB binding sites with the promoter region. These results suggest that the anti-inflammatory effects attributable to LXR activation were mediated through inhibition of NF-kB signalling. However, similar studies analysing the role of LXR activation in human macrophages demonstrated a bi-modal effect of LXR activation. Pre-incubation of primary LPS stimulated monocyte derived macrophages with LXR agonists for less than 24

hours inhibited the secretion of TNFα and MCP-1. In contrast cytokine and chemokine release was exacerbated when pre-incubation of macrophages with T1317 was greater than 24 hours (61). Similar results have also been obtained in primary human pulmonary derived macrophages (118). Further studies demonstrated an LXRE within the human TLR4 promoter and that the increased cytokine secretion in response to TLR4/ LXR ligation was mediated by the increased induction of TLR4 expression. These observations therefore demonstrate a species difference in the response to endotoxin in the context of LXR activation between mouse and humans. To date no other studies have analysed the effect of LXR activation upon other pattern recognition receptors (PRRs) in mice or humans and whether the regulation of human TLR4 by LXRs is unique or is a characteristic of other human TLRs is unknown.

The potential for LXR activation to modulate an immune response has been assessed in pulmonary inflammation. Two studies have independently demonstrated that induction of pulmonary inflammation with an LPS aerosol was significantly attenuated by systemic administration of T1317 or GW3965. This was associated by a reduction of iNOS and IL-1β expression and reduced neutrophil infiltration into the trachea (19, 118). However, the inhibition of cytokine expression and neutrophil recruitment by LXR activation was shown to have detrimental consequences upon intra-tracheal infection of mice with the gram negative bacterium *K. pneumoniae* where the number of lung and spleen colony forming units was increased leading to an increased level of mortality. However, in an endotoxin free antigen driven model of asthma administration of GW3965 significantly increased airway smooth muscle proliferation, reactivity and eosinophilia (119). Together these studies suggest that the inflammatory context in which LXRs are activated will dictate whether a beneficial or detrimental outcome is achieved. Furthermore, LXR activation should therefore be described as having an immune modulatory potential as opposed to the current anti-inflammatory role which LXRs are generally ascribed.

LXR agonism has also been shown to inhibit cytokine release and proliferation of antigen stimulated murine $CD4^+$ T cells *in vitro* demonstrating a potential role for LXRs in the adaptive immune response (17, 120, 121). Accordingly, the effect of LXR activation has been investigated in rodent models of human autoimmune disease. Treatment of mice with T1317 ameliorated the severity of an *in vivo*

model of human multiple sclerosis (experimental autoimmune encephalomyelitis - EAE) (121, 122). However, *ex vivo* analysis demonstrated that this was not associated with a decrease in antigen driven T cell proliferation contradictory to preliminary *in vitro* observations (121). Furthermore, gene expression analysis of human peripheral blood mononuclear cells (PBMCs) from patients with MS showed altered levels of LXR expression supporting a role for LXRs in human disease pathology. Interestingly, the changes in LXR expression were shown to differ between different ethnic and geographical groups (123, 124). It is therefore clear that the expression of LXRs and potentially their activity is influenced through genetic and/ or environmental differences although the specific factors and their magnitude of effect are currently unknown.

More recently the effects of LXR activation have also been assessed in the murine collagen-induced arthritis (CIA) model. Administration of T1317 at high dose after antigen challenge was shown to significantly inhibit disease progression and was therefore suggestive of a beneficial effect of LXR agonists for the treatment of rheumatoid arthritis (RA) (125). However, my work presented within this thesis shows a clear contradictory outcome; I have demonstrated that administration of either T1317 or GW3965 dramatically increase the severity and onset of disease progression in multiple CIA models. The results of my studies and an in depth comparison of these studies will be presented and discussed in the respective chapters contained herein.

6.2.7 Liver X Receptor agonists as therapeutics for disease

It is evident from studies in rodents that synthetic LXR agonists may exert potential beneficial effects upon metabolic related pathologies. Several pharmaceutical companies are competing to develop LXRβ agonists that will mediate these potentially beneficial effects without inducing LXRα activation which is implicated in fatty acid and triglyceride synthesis in turn promoting the development of hepatic steatosis. Recently, a novel LXRα/ LXRβ dual agonist, WAY-252623, was tested in a human recipient and was shown to up-regulate ABCA1 expression in PBMCs. These results suggest that the ability of LXR activation to induce reverse cholesterol transport in mice, the primary mechanism of atherosclerotic lesion size reduction by LXRs, is conserved

between rodents and humans *in vivo* (36). Furthermore, this study provided the first evidence that LXRs agonists may indeed be an appropriate therapeutic approach for the treatment of human metabolic disorders. The safety of WAY-252623 has since been tested in a phase I dose ascending study in a total of forty volunteers (126). The agonist was shown to be readily absorbed and to induce the expression of ABCA1 and ABCG1 in PBMCs; suggestive of sufficient exposure of LXRs to agonist at all concentrations by oral administration. However, whilst no deaths or serious adverse effects were reported, 55% of the participants experienced mild adverse effects of a psychiatric or neurological nature during and post termination of their assigned treatment regime. Some of the symptoms included forgetfulness, drowsiness and confusion. From this single study it is not clear if these adverse effects were a result of agonist toxicity, lack of specificity or alteration of LXR function and downstream pathways within the brain. It is however clear that further studies are required to elucidate the mechanism of these effects if LXR agonists are to go forward to the clinic.

The role of LXRs in inflammation remains unclear and at this stage it is not clear whether development of LXR agonists would be useful in human inflammatory diseases. This is particularly relevant since unexpected events could occur through the modulation of inflammatory pathways associated with metabolic disease.

6.3 Rheumatoid arthritis

6.3.1 Diagnosis

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease that affects 1% of the population of which approximately 70% of patients are female. RA presents as polyarthritis that predominantly affects the hands, feet and wrists but can affect any diarthrodial synovial joint. Diagnosis of RA requires the presence of four or more of the 1987 American College of Rheumatology (ACR) criteria for the classification of RA (Table 6.2) (127). These symptoms must persist for at least six weeks to exclude confounding conditions, such as viral infection, for a clinician to be confident of the correct diagnosis. Unfortunately this also presents a period of time in which symptoms may progress untreated. Novel criteria for the classification and diagnosis of RA that recognise these deficiencies are in the process of being prepared and will be available in early 2010 under the direction of EULAR and the ACR. RA is extremely debilitating - approximately 80% of patients have reduced functional capacity to varying degrees and RA is therefore associated with a high social burden and economic cost due to unemployment (128). Therefore, identifying the underlying cause of RA and developing remission inducing treatment strategies is essential.

Criteria	Definition
Early morning stiffness	Stiffness of the joints lasting at least one hour
Arthritis of ≥ 3 joints	Three joint areas that exhibit joint swelling
Hand joint arthritis	Arthritis of at least one area of the hand/ wrist
Symmetrical arthritis	Arthritis of the same joint areas on either side of the body
Rheumatoid nodules	Subcutaneous non tender nodules generally found at pressure points near joints
Rheumatoid factor (RF)	Presence of an autoantibody against self IgG
Bone erosions	Radiographic changes typical of rheumatoid arthritis on hand and wrist including erosions or bone decalcification

Table 6.2 American College of Rheumatology (ACR) criteria for classification of rheumatoid arthritis.
Diagnosis of rheumatoid arthritis requires the presence of at least four of the ACR criteria to be diagnosed by a clinician (127).

6.3.2 Etiology of rheumatoid arthritis

Although how RA is initiated remains unknown there are a number of factors, both genetic and environmental, which are known to contribute to the susceptibility of developing RA. These risk factors may confer an increased risk of developing RA whilst others may offer protection. Similarly, others factors may exert modulatory effects upon the disease once onset. Whether protective or causative identifying these risk factors and understanding how they impact upon pathogenesis is vital to identifying the cause of RA.

6.3.2.1 Genetic associations

Twin studies suggest that the genetical heritability of RA is approximately 50% in the UK (129). The most striking genetic association is with the various HLA-DRB1 and HLA-DR4 alleles within the MHC class II region on human chromosome 6 (130). Several HLA-DRB1 genotypes have been shown to confer a higher risk of early disease onset whilst others were associated with delayed onset and were therefore suggested to be protective (131). Association of MHC class II haplotypes with RA has given rise to the shared epitope hypothesis (132). The shared epitope hypothesis suggests that mutations in the β chain of the MHC class II molecules may alter T cell receptor (TCR) selection in the thymus, the recognition of self or modified self peptide by antigen presenting cells or the way in which antigen/ MHC class II interacts with the TCR to drive an immune response. However, it is evident that not all individuals with these polymorphisms develop RA and therefore there must be other contributing factors. Genome wide association studies have been utilised to identify further genetic risk factors of RA in which genomic markers adjacent to candidate genes such as STAT4, TNFα receptor and PTPN22 were identified as being disease associated (130). These observations imply that there are multiple pathways that may contribute to potentiate the development of RA. Theoretically, by understanding the genetics of RA, treatment regimes may be tailored towards individuals with a particular genetic predisposition. For example it is proposed in one study that mutations within the gene encoding TNFα may predict a poor response to antibody mediated anti-TNFα therapies (Infliximab) but not soluble TNFα receptor mediated therapy (Etanercept) (133). Many other studies are ongoing to clarify such an approach particularly in predicting response to biologic therapeutics. However, as susceptibility to RA is also largely influenced by environmental variation it is unlikely at present that genetic screening alone will be useful for the pre-clinical diagnosis of RA.

6.3.2.2 Environmental impact

As the genetic heritability of RA has been estimated to be approximately 30-50% it is evident that environmental risk factors confer a large influence upon the susceptibility to RA. In particular cigarette smoking, which is of a higher incidence in RA populations, has been one of the most widely studied

environment risk factors (134). Epidemiological studies have demonstrated that smoking is associated with a thirteen fold increase in the incidence of RA and an increased severity of disease (134-136). Furthermore, smoking induces long term susceptibility as individuals who have stopped smoking for more than ten years remain twice as likely to develop RA than the general population (137). Cigarette smoking promotes the process of citrullination; the post-translational modification of the amino acid arginine by de-imination to citrulline is catalysed by the enzyme peptidylarginine deaminase 4 (PAD 4). Citrullinated proteins including type II collagen, α-enolase, fibronectin, vimentin and fibrin have been identified in RA synovium and have been shown to be targets of an autoantibody response (138-140). Indeed, anti-citrullinated protein antibodies (ACPAs) have been identified in up to 90% of patients with RA and although they are not absolutely specific to RA they do correlate with disease severity (141-143). Citrullinated proteins are more immunogenic and have been suggested to contribute via the shared epitope hypothesis by modifying self peptide which may drive an immune-response (143). In support of this there is a strong association of smoking with “pathogenic” HLA-DRB1 alleles and the production of ACPAs in RA, thereby providing an example of how genetic and environmental factors may interact to drive pathology in RA (144).

6.3.3 RA synovium

The primary site of inflammation in RA is the synovial membrane (synovium). In a non-inflamed joint a protective smooth layer of cartilage acts as a “shock absorber” and provides a low friction surface to allow the bones to slide over each other. This is lubricated by synovial fluid which in addition is also a source of nutrients to chondrocytes as cartilage lacks blood vessels. A fibrous and ligamentous capsule around the joint is lined by the synovial membrane that is normally one to three cell layers thick comprising a thin lining layer of predominantly synovial fibroblasts and some macrophages with underlying adipose cells and a few blood vessels. RA is characterised by articular destruction and inflammation mediated by inflammatory infiltration of the synovial membrane (synovitis). This in turn drives joint pain and stiffness representing typical symptoms. Radiographic examination reveals decreased joint space and loss of cortical bone integrity i.e. cartilage and bone erosion,

limiting articulation and promoting further bone erosion potentially via biomechanical pathways. Articular destruction is accompanied with synovial membrane hyperplasia, pannus formation (intrusion by proliferation of the synovial membrane into the cartilage and bone interface) and inflammatory cell infiltration into the synovium. Infiltrating leukocytes promote synovitis by secreting cytokines, chemokines and proteases which mediate cartilage and bone erosion and promote a state of hypoxia. Hypoxia in turn induces angiogenesis of the synovial membrane and may actually enhance the survival of some inflammatory cells e.g. neutrophils (145). The comparison between a normal healthy and an inflamed RA synovium is illustrated in Figure 6.6.

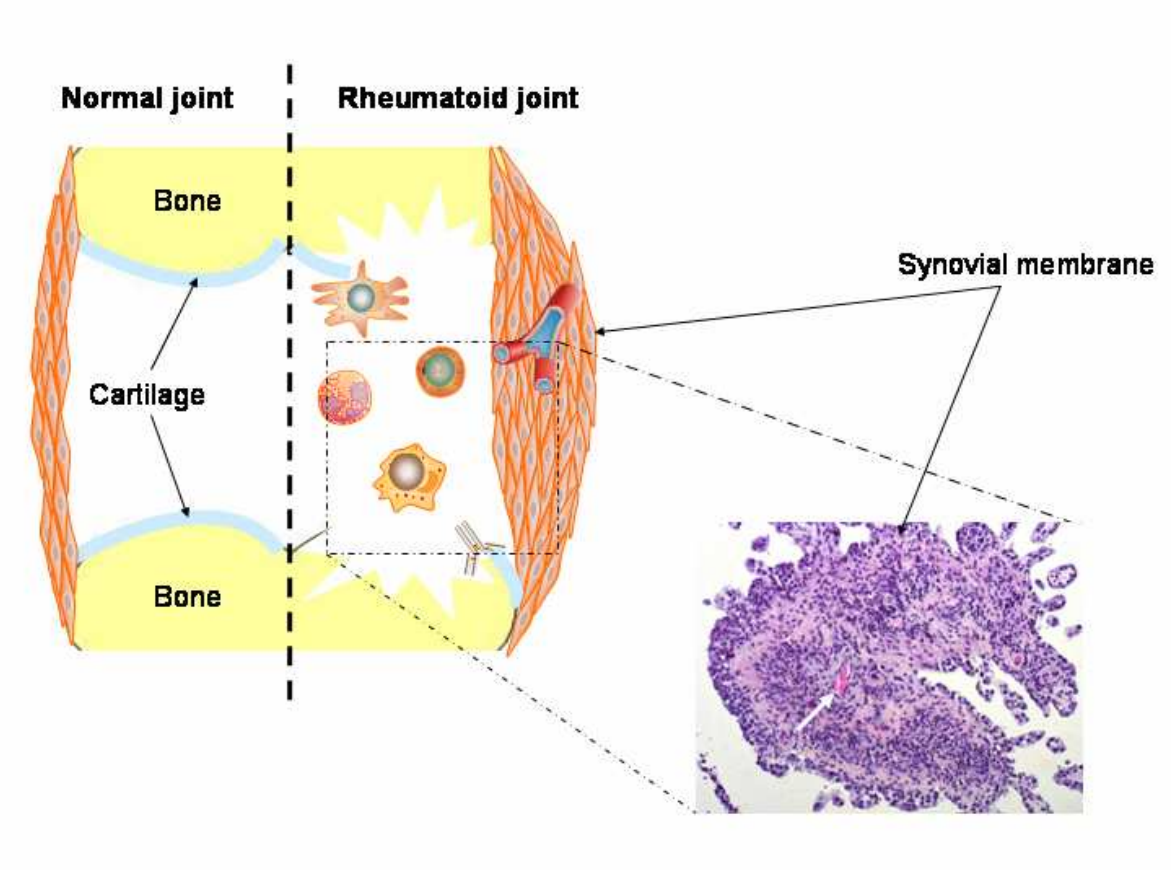


Figure 6.6 Rheumatoid Synovium
Comparison of a normal synovial joint compared to an inflamed rheumatoid arthritis synovial joint. The illustration demonstrates the presence of inflammatory cell infiltrates synovial membrane hyperplasia, cartilage and bone erosion and angiogenesis. The histology photograph is a representative Haematoxylin & Eosin stained rheumatoid synovial membrane. The white arrow indicates a blood vessel (photo kindly supplied by Dr Axel Hueber).

6.3.4 Inflammatory cell types of the synovium

It is evident that synovitis is accompanied by an inflammatory cell response which drives tissue destruction and inflammation. In order to develop an understanding of the processes which drive synovitis it is important to firstly identify which cells are present within the synovium and elucidate how they might contribute to the ongoing inflammatory response. The interactions between inflammatory cells is summarised in Figure 6.7.

6.3.4.1 The monocyte/ macrophage lineage

Monocytes are derived from common myeloid progenitor cells within the bone marrow. Myeloid progenitors firstly differentiate into monoblasts and subsequently pro-monocytes which upon migration into the blood give rise to monocytes. However, the monocyte population is not homogenous and in mice they have been separated into two general categories; inflammatory monocytes ($GR1^+$, $CCR2^+$ and CX_3CR1^-) and blood resident monocytes ($GR1^-$, $CCR2^-$ and CX_3CR1^+) (146). Similarly, human monocytes can be categorised as classical monocytes ($CD14^+CD16^-$) which are analogous to the murine inflammatory monocytes and account for approximately 90% of monocytes in the blood (146, 147). Additionally, approximately 10% of human monocytes are described as non-classical monocytes ($CD14^+CD16^+$) akin to the murine blood resident monocytes. These two monocyte populations give rise to two broad classes of macrophage populations, termed M1 and M2, which exert different effects during an inflammatory response (discussed below).

Classically activated/ effector macrophages (M1) differentiate from classical/ inflammatory monocytes and are found within the inflamed synovial membrane in RA. They are characterised by the expression of inducible nitric oxide synthase (iNOS) and are generally associated with bacterial clearance and exert pro-inflammatory effects (148). Differentiation of M1 macrophages is induced upon exposure to the Th1 cytokine IFNγ in combination with TNFα, both of which are elevated in patients with RA. M1 macrophages can also be differentiated in an autocrine dependent manner through the secretion of TNFα and IFNß upon stimulation with Toll Like Receptor (TLR) ligands e.g. LPS in response to bacterial infection. Furthermore, TLR ligands such as bacterial cell wall

fragments, peptidoglycan and double stranded DNA have been identified within the synovium of RA patients which may drive polarisation of M1 cells (149). The expression of TLR3 and TLR4 are enhanced in synovial tissue from a proportion of patients with RA relative to osteoarthritis (OA) (150). Administration of LPS in the murine model of CIA also dramatically increases the severity of disease and re-initiates disease in mice during remission (151). Additionally, inhibitors of TLR7/8 have been shown to reduce TNFα secretion from RA synoviocytes (152). This has lead to suggestions that RA may be initiated by bacterial and or viral infection although to date no microorganism has been specifically linked to pathogenesis. However, it is now recognised that TLRs can be activated by endogenous ligands such as heat-shock proteins and double stranded RNA released from necrotic synovial cells (153). Therefore, it is possible that the local environment within the synovium may potentiate the inflammatory response through a TLR mediated pathway by the secretion of pro-inflammatory cytokines, e.g. IL-12, IL-23 and TNFα, and activation of other immune cells e.g. T cells and fibroblasts. Future studies will almost certainly implicate further pattern recognition receptors e.g. NOD family members.

The effector pathways mediated by classical synovial macrophages are well defined and include the secretion of cytokines and chemokines (e.g. TNFα, IL-1, IL-6, MIP-1α and MCP-1) and matrix metalloproteinases (e.g. MMP9 and MMP12). Additionally, macrophages can induce the activation of other adjacent cells by cell contact such as fibroblasts to induce the secretion of GM-CSF, IL-6 and IL-8. Although macrophages are not considered as professional antigen presenting cells such as dendritic cells and B cells, are it is clear that they can process and present peptides through MHC class II to induce the activation of $CD4^+$ T cells. Such cellular interactions between macrophages and T cells enforces the inflammatory response within the RA synovium. Macrophages can also interact with and subsequently induce the activation of endothelial cells. This has been suggested to be in part responsible for the sustained influx of inflammatory cells into the synovium. Additionally, activated macrophages in RA may also drive atherosclerotic plaque development through the interactions with vascular endothelial cells. However, as professional phagocytes macrophages also serve to resolve inflammation through the phagocytosis of neutrophils and the secretion of anti-inflammatory cytokines such as IL-10 and TGF-ß.

Although the function of the non-classical/ resident monocytes is poorly understood they have recently been described as patrolling monocytes since they migrate along the luminal surface of blood vessel walls (154). These cells are ideally situated for immuno-surveillance and rapidly extravasate upon surrounding tissue damage and bacterial infection to provide a rapid and early but short lived (< 8hrs) inflammatory response. These monocytes were also characterised by the induction of arginase 1 and mannose receptor (CD206) expression which is typical of alternatively activated (M2) macrophages. M2 macrophages can be differentiated *in vitro* upon exposure to the Th2 cytokines IL-4 and IL-13 and are generally associated with a wound healing response and an anti-inflammatory function (148). Although M2 macrophages do not appear to have a dominant role in bacterial clearance they have been implicated in the clearance of nematode parasite infections (155). To date the presence of alternatively activated macrophages within the synovium, either in a healthy or pro-inflammatory environment, has not been demonstrated and their potential role is unknown. However, providing they are present in the first instance, it is likely that during propagating stages of synovial inflammation that they are differentiated towards or replaced by macrophages exerting a pro-inflammatory phenotype.

The role of M1 and M2 macrophages has been studied in the pathogenesis of many inflammatory disorders. However, it is now evident that these M1 and M2 phenotypes represent two extremes of the macrophage activation spectrum that is in fact rather plastic (148). Furthermore, the monocyte lineage is highly heterogeneous and upon migration into tissues they will differentiate according to the external signals they receive. This is of importance in RA since ligation of receptor activator of NF-kB ligand (RANKL) expressed on synovial fibroblasts and T cells with its cognate receptor on monocytes, RANK in the presence of M-CSF induces the differentiation of osteoclasts. These are multi-nucleated cells that promote bone resorption and are responsible for the degradation of bone commonly seen in RA. Thus monocyte lineages can not only promote inflammation by virtue of cytokine and enzyme release but can also contribute directly to the resorption of bone by alternate differentiation.

It is evident that monocytes and macrophages are central to the cellular responses involved in the pathogenesis of RA. Understanding the signals that

induce monocyte/ macrophage differentiation and the subsequent implications upon their activities is therefore crucial to understanding the development of RA pathology.

6.3.4.2 Dendritic cells

As well as giving rise to monocytes myeloid progenitor cells can also differentiate into dendritic cells (DCs) - classical/ inflammatory monocytes may also be able to replenish specific dendritic cell populations e.g. Langerhans cell in the skin. DCs are highly phagocytic and endocytotic cells that are able to both recognise and respond to microbial infection and thereafter present antigen and are therefore central to the cross-talk between the innate and adaptive immune response. DCs are found in tissues in an inactive state however upon immune-activation, either by TLR ligands or antigen uptake they down-regulate their phagocytic capacity but up-regulate antigen processing and presentation and are directed to lymph nodes via CCR7 dependent pathways to induce T cell activation (156). Two major subsets of dendritic cells have been identified; plasmacytoid dendritic cells (pDCs) and myeloid dendritic cells (mDCs). mDC have a dominant role in antigen uptake and subsequent presentation. They express a large variety of TLR and NOD receptors and rapidly sense tissue damage or 'danger'. They predominantly produce cytokines such as IL-12, IL-23, IL-15, IL-18 and by this means mediate significant effects on subsequent T cell differentiation. pDC in contrast have equivocal antigen presenting function but particularly upon viral infection are a major source of type I interferon synthesis and release. In RA circulating numbers of mDC and pDC are both reduced and both correlate with disease activity inversely as assessed by correlation with CRP (157). Depletion of murine pDCs *in vivo* has recently been shown to enhance an anti-collagen response suggesting that they mediate mainly anti-inflammatory or regulatory effects (158, 159). mDCs in contrast when transferred into recipient mice and pulsed with collagen induce erosive arthritis and as such they seem likely to exert pro-inflammatory effects in arthritis (159).

6.3.4.3 Synovial T cells

$CD4^+$ effector T cells can be divided into distinct lineage subsets depending upon their cytokine secretion profile. Th1 cells are categorised by the secretion of the

pro-inflammatory cytokine IFNγ and IL-2 which is required for T cell proliferation whereas Th2 cells are typified by the secretion of the cytokines IL-4, IL-5 and IL-13. In addition a new subset of $CD4^+$ T cells has recently been described termed Th17 due to their secretion of the pro-inflammatory cytokine IL-17A, together with IL-17F, IL-22 and CCL20.

Cytokines secreted by activated macrophages regulate T cell activation and survival; thus IL-12 secretion from M1 macrophages or mDCs induces the polarisation of naïve $CD4^+$ T cells towards a Th1 IFN-γ secreting phenotype. Th1,$CD4^+$ $IFN\gamma^+$, cells are detectable in synovial membrane at an elevated ratio to Th2 cells (160). In addition to IFNγ and IL-2, ligation of the T cell receptor (TCR) induces the secretion of TNFα and potentially a variety of other cytokines. However, whilst synovial T cells secrete TNFα a proportion also secrete IL-10 and only a very small number are found to express IFNγ in situ in the tissue (161). Therefore only a proportion of synovial T cells fit the classical Th1 cytokine secretion profile.

Synovial T cells were shown to have a similar phenotype to cytokine activated T cells (TcKs). These can be generated *in vitro* by treatment with IL-6, TNFα and IL-2 which when co-cultured with M-CSF matured macrophages induce the secretion of multiple macrophage derived pro-inflammatory cytokines including TNFα (162). IL-2, IL-6 and TNFα are all detectable at elevated levels within the synovium and histological analysis of synovial membrane has revealed that macrophages and T cells are within close proximity to each other. In accordance co-culture of synovial membrane derived T cells with macrophages was shown to induce the secretion of macrophage derived TNFα in a cell-contact dependent manner independent of TCR ligation. In comparison treatment with IL-15 alone has also been shown to induce proliferation and activation of T cells akin to the cytokine cocktail IL-2, IL-6 and TNFα. Furthermore, co-culture of IL-15 activated T cells induced macrophage derived secretion of TNFα (163). IL-15 is expressed primarily by macrophages, fibroblast like synoviocytes and endothelial cells and is found at elevated levels in the serum and synovial fluid of RA patients relative to osteoarthritis (OA) patients (164). Serum and synovial fluid concentrations correlate strongly to disease severity, CRP and DAS28, in established disease thus suggesting a central role in disease pathogenesis (165).

IL-15 has also been shown to induce the secretion of IL-17 from PBMCs and RA synoviocytes (166). IL-17 is found at elevated levels in RA synovial fluid and was first shown to be produced by $CD4^+$ T cells termed Th17 cells (167). Deletion of IL-17 or administration of an anti IL-17 antibody markedly reduced the severity of CIA whereas overexpression of IL-17 exacerbated disease severity (168, 169). As noted above however, Th17 cells are present in low numbers in inflamed RA synovium (170). Differentiation of $CD4^+$ T cells towards a Th17 phenotype can be induced *in vitro* by IL-21, IL-6 and TGF-ß whilst the expansion of Th17 cells is mediated by IL-23. However, the frequency of Th17 cells is low and Th1 cells may even predominate within the synovium (171). It is not clear whether this represents plasticity of the Th17 lineage or differentiation of a proportion of naïve T cells present within the heterologous Th17 population. However, Th17 cells also secrete IL-22 and CCL20 and in humans a proportion of Th17 cells are $IFN\gamma^+$ (172). Additionally, it is now evident that IL-17 is also secreted by non $CD4^+$ T cells; specifically NK cells, γδ T cells, CD8 T cells, macrophages and more recently mast cells (unpublished data Hueber *et al*) (173-175). It is therefore unlikely that the observed phenotype upon deletion of IL-17 or IL-17 blockade is completely attributable to Th17 cells, contrary to initial suggestions, but rather a combination of cellular sources. Secretion of IL-17 drives activation of monocytes/ macrophages and synovial fibroblasts to secrete a milieu of pro-inflammatory cytokines and chemokines e.g. IL-1, IL-6, IL-8, TNFα and GM-CSF (176). Blockade of IL-17 is therefore expected to be beneficial and is an attractive therapeutic target for the treatment of RA.

In addition to the Th1, Th2 and Th17 subsets is the $CD4^+$, $CD25^+$ regulatory T cell lineage(s) (Tregs) that express forkhead box p3 (Foxp3). Unlike $CD4^+$ effector T cells which promote an inflammatory reaction the role of Tregs is to regulate effector T cell activation and are therefore generally ascribed an anti-inflammatory property. Tregs have been shown to suppress inflammation through direct cell-cell contact and by the secretion of the anti-inflammatory cytokines IL-10 and TGF-ß. However, the precise mechanisms by which Tregs mediate inflammatory suppression are not fully understood. Although Tregs are detectable in peripheral blood and synovial fluid of patients with RA they have a decreased capacity to suppress IFNγ production from $CD4^+$ T cells (177). Intriguingly the ability of Tregs to inhibit effector T cell IFNγ secretion was

shown to be mediated through TNFα; as treatment of RA patients with anti-TNFα antibodies (Infliximab) restored the capacity of Tregs to suppress pro-inflammatory cytokine secretion. Thus the synovial inflammatory environment may inhibit the action of Tregs and thereby potentiate effector T cell proliferation and subsequent monocyte/macrophage activation through enhanced cytokine secretion.

$CD8^+$ T cells are typically described as cytotoxic T cells due to their ability to recognise virus infected cells and induce programmed cell death (apoptosis). This is mediated by the release of lytic granules which form pores in plasma membranes and digest proteins and nucleic acids. Additionally $CD8^+$ T cells also secrete IFNγ which inhibits virus replication and increases the expression of MHC class I. $CD8^+$ T cells are found diffusively spread within the RA synovium (178). Interestingly whilst the number of $CD4^+$ T cells was shown to positively correlate with leukocyte infiltration the frequency of $CD8^+$ T cells was inversely associated with synovial membrane leukocyte numbers. This therefore suggests that $CD8^+$ T cells could promote or regulate an inflammatory response within the RA synovium. However, the role of CD8+ T cells in RA is unclear and reports are conflicting as $CD8^+$ T cells have also been suggested to modulate formation of germinal centres (179, 180).

6.3.4.4 B cells

RA is associated with autoantibody production against self citrullinated proteins (ACPA) and IgG (rheumatoid factor - RF) which are detectable in serum up to 10 years prior to the clinical onset of RA (181). Additionally, B cell depletion by the anti-CD20 targeting monoclonal antibody, rituximab, has been successful in ameliorating disease in a large number of patients with RA (discussed below). It is therefore evident that B cells are central to driving the pathology of RA. B cells drive the inflammatory response in part through antigen presentation thereby inducing T cell activation and proliferation. Upon activation by antigen B cells will migrate to the lymph nodes or spleen where they will differentiate into antibody secreting plasma cells. Auto-antibody production can then induce the activation of inflammatory cells through binding of the Fc portion of antibodies to Fc receptors expressed on leukocytes. The importance of antibody mediated immune cell activation has been confirmed by the induction of

arthritis through the transfer of serum from an immunized mouse into a non-immunized recipient in which type II collagen is thought to be the dominant epitope (182). Furthermore, anti-collagen antibody cocktails have been shown to induce the development of collagen-antibody induced arthritis (CAIA) which is characterized by macrophage and polymorphonuclear inflammatory cell infiltrate (183). Finally B cells may act as a source of inflammatory cytokines in RA synovial membrane and promote inflammation by that route.

Analysis of synovial membrane has revealed the presence of lymphoid like structures in a proportion of patients with RA (184, 185). These structures, which resemble lymph node germinal centres, range from loose T and B cell aggregates to highly organised lymphoid like structures. These ectopic germinal centres are in an environment surrounded by self antigen and pro-inflammatory cytokines; they therefore provide an ideal setting for antigen presentation, plasma cell differentiation, survival and auto-antibody production without the requirement for trafficking to peripheral lymph nodes (186). However, it is difficult to assess the role that ectopic germinal centres play in the pathology of RA. Most samples obtained are small biopsies which consist of only a small proportion of the total potentially involved synovial membrane and therefore determining even a correlation between the presence of ectopic germinal centres relative to disease severity has proven problematic. Further, studies are therefore required to define the role of such lymphoid structures upon B cell activation and how this may affect the pathology of RA.

6.3.4.5 Other inflammatory cells

Mast cells & Neutrophils

In addition to lymphocytes, DCs and macrophages, mast cells are also thought to play a role in RA and are present within the RA synovium at sites of cartilage damage (187). However, arthritic protocols in mice to study the role of mast cells upon the initiation of inflammation have yielded conflicting results (188, 189). Whereas it is uncertain if mast cells are crucial for the initiation of arthritis it is evident they can mediate disease severity (190). However, unlike mast cells, depletion of neutrophils prevents the onset of adjuvant induced arthritis suggesting a role for neutrophils in the effector phase of RA (191). Neutrophils are concentrated within synovial fluid which in part is due to the

nature of the synovial environment as synovial fluid in conjunction with hypoxia enhance neutrophil survival (145). Stimulation of fibroblasts with TNFα and IL-17, two cytokines central to RA pathology, induces the secretion of GM-CSF which can promote the survival of neutrophils (192).

Fibroblasts

Fibroblasts are the major cell type of the synovial membrane and it is well established that they contribute towards pathology in RA. Activation of fibroblasts either by cytokines e.g. GM-CSF or by TLR ligands induces the secretion of a variety of cytokines including IL-1, IL-6, TNFα and M-CSF which can in turn potentiate the survival and activation of leukocytes such as macrophages. The secretion of inflammatory cytokines by fibroblasts also serves as an auto-regulatory loop to upregulate the expression of adhesion molecules ICAM-1 and VCAM-1 promoting the interaction of fibroblasts with T cells (193). Such interactions enforce the inflammatory response through the increased expression and secretion of pro-inflammatory cytokines such as IL-1. The secretion of pro-inflammatory cytokines by fibroblasts and other immune cells also induces the secretion of chemokines, e.g. CXCL2 and CCL8, which can induce the chemotaxis of neutrophils and monocytes/macrophages respectively into the synovium. It is therefore evident that fibroblasts are central to the inflammatory response in the RA synovium. Additionally, fibroblasts are also able to contribute towards articular destruction directly through the secretion of proteinases e.g. collagenase (MMP-1). RA fibroblasts also express higher levels of RANKL and therefore drive articular destruction indirectly through the induction of osteoclastogenesis (194).

Osteoclasts & Chondrocytes

Articular structure in healthy individuals is maintained in a stable equilibrium by which the rate of bone deposition and resorption and collagen synthesis and degradation is in balance. However, in the RA synovium this balance is skewed towards bone resorption and collagen destruction leading to articular destruction. Osteoclasts are the cells which mediate bone resorption which is necessary for skeletal remodelling during development but are also the major cause of bone digestion in RA. Osteoclasts are multi-nucleated cells differentiated from monocyte precursors through the interaction of RANK/

RANKL. Osteoclast activation and differentiation can be enhanced through the actions of cytokines such as TNFα or through cellular interactions where by T cells or fibroblasts can promote the differentiation of monocytes to osteoclasts (194). Osteoclasts degrade bone through the secretion of acid and MMPs which can cleave matrix proteins such as collagen. Similarly collagen turnover is also regulated in part by the actions of MMPs although this process is primarily mediated through the actions of chondrocytes. The secretion of MMPs, and other proinflammatory mediators e.g. IL-17 and IL-6, from chondrocytes can be induced by multiple pro-inflammatory cytokines such as IL-1 and TNFα (195). Therefore, it is evident that the inflammatory environment within the RA synovium can promote articular destruction through several mechanisms.

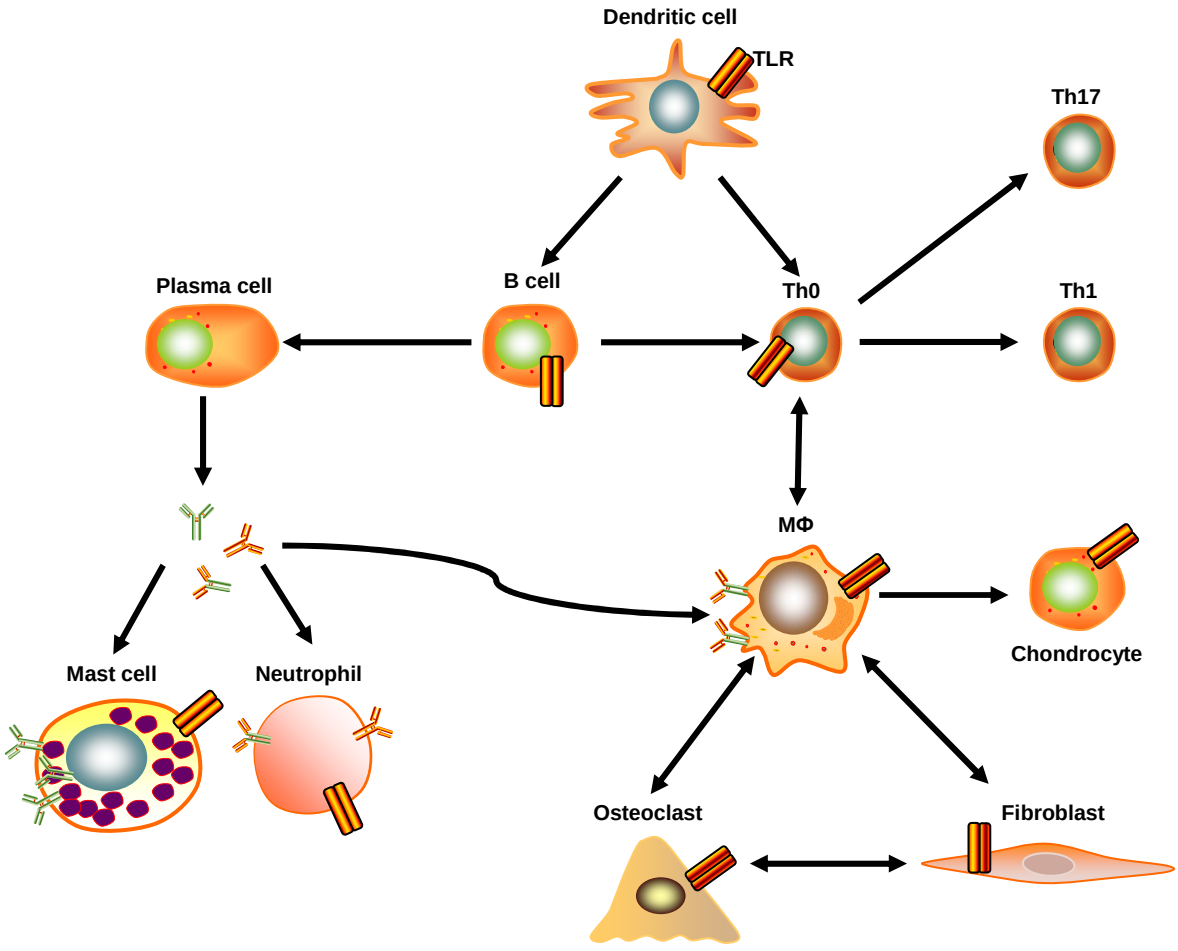


Figure 6.7 Inflammatory cell types of the synovium
Schematic demonstrating the interactions of immune cells within an inflamed joint, adapted from (196).

6.3.5 Cytokines & chemokines in rheumatoid arthritis

Neutralisation of cytokines is now an established therapeutic regime exemplified by the use of anti TNF-α antibodies and soluble receptor fusion proteins in the clinic. Therefore, understanding the impact of cytokine networks upon inflammation and identifying how they are regulated is central to elucidating the mechanisms by which disease progresses (summarised in Table 6.3).

6.3.5.1 TNFα

TNFα is detectable in synovial fluid and membrane and is secreted by monocytes/ macrophages, T cells, B cells, synovial fibroblasts and neutrophils upon cytokine activation, TLR ligation or cell-cell interactions. Binding of TNFα to its cognate receptor TNF receptor 1 (TNFR1) induces activation of NF-κB and the secretion of multiple pro-inflammatory cytokines, e.g. IL-1 and IL-6, from synoviocytes which is blocked by addition of anti-TNFα antibodies *in vitro* (197, 198). As such TNFα is now considered to be at the top of the cytokine hierarchy, or at least critical to the regulation of such a hierarchy. Several *in vivo* studies demonstrated a pathogenic role for TNFα in arthritis. Transgenic mice expressing human TNFα spontaneously develop arthritis and administration of anti-TNFα blocking antibodies ameliorated the severity of disease in a dose responsive manner (199, 200). Since these observations several TNFα blocking agents have been developed for the treatment of human RA (discussed below).

6.3.5.2 Interleukin-12 superfamily

Members of the IL-12 superfamily of cytokines are related by their common sub-units p35, p40 and p19 and are secreted primarily by macrophages and dendritic cells upon stimulation by cytokines or TLR activation. IL-12(p35/p40) plays a pivotal role in the development of Th1 responses by promoting the polarisation of naïve $CD4^+$ T cells towards an IFN-γ secreting phenotype. The role of IL-12 in RA is unclear however low levels of IL-12(p35/p40), i.e. biologically active IL-12, are detectable in RA synovial membrane and may therefore support the expansion of the predominant Th1/ TcK population within the RA synovium (201). IL-12 is considered to be a pro-inflammatory cytokine. However mice deficient in IL-12p35 display increased severity of CIA, associated with enhanced

levels of multiple pro-inflammatory cytokines and chemokines including IL-6, IL-17 and TNF-α, suggesting IL-12 is protective in this model (202). In contrast, studies in other models, e.g. EAE, have demonstrated beneficial effects attributable to targeted deletion of the gene encoding the p40 sub-unit (203). However, it is now recognised that the p40 sub-unit is shared with IL-23(p19/p40). Therefore such beneficial effects may be at least in part elicited by IL-23 blockade (203, 204). Mice deficient in p19 exhibit reduced severity of CIA (202). Interestingly IL-12 is overexpressed in mice deficient for IL-23 suggesting a mechanism of IL-12/ IL-23 cross regulation. IL-23(p19) has been detected at high levels in the serum and synovial fluid and is overexpressed by synovial fibroblasts in RA (205). Similarly, we have demonstrated IL-23(p19) expression by immunohistochemistry in RA and PsA synovial membrane. However, functional IL-23p19/p35 heterodimer was only detectable by ELISA in PsA, but not RA, synovial fluid (unpublished data from our group) suggesting that at least in established disease and within the synovium IL-23 does not have a pathological role in RA. The role which IL-23 plays in early disease is unknown however, as well as promoting Th17 cell survival IL-23 has been shown to induce the secretion of pro-inflammatory cytokines e.g. IL-6, TNFα and IFNγ from T cells (206).

6.3.5.3 Interleukin - 17

The IL-17 family consists of six members, IL-17 A through to F, of which IL-17A (commonly referred to as IL-17) has been the most widely studied. IL-17 signals through the IL-17 receptor A (IL-17RA) which is ubiquitously expressed. The secretion of IL-17 has been shown to induce the expression of IL-6 and IL-8 in mouse fibroblasts and promote neutrophil survival (207). A pathological role for IL-17 in the development of arthritis has now been demonstrated in the murine CIA model as previously mentioned. Furthermore, mice deficient in IFNγ were more susceptible to the development of CIA which was suggested to be due an unrestricted expansion of the Th17 cell population mediated by loss of IFNγ (208). Given the highly pathological role of IL-17 there has been a concerted effort to elucidate the biology of IL-17 with particular emphasis upon Th17 cells; the first identified cellular source of IL-17; although as discussed above multiple sources have since been identified. Given the necessity of IL-6 for Th17 cell differentiation targeting of IL-6 may inhibit Th17 cell polarisation whilst

favouring the development of regulatory T cells mediated by TGFβ and thereby promote a protective immune response.

6.3.5.4 Interleukin- 6

IL-6 is expressed by monocytes, lymphocytes and fibroblasts and is detectable at elevated levels in the serum and synovial tissue of RA patients. Serum concentrations of IL-6 correlate with levels of CRP and disease severity (209). This strongly suggests a central role in disease pathogenesis and supports the suggestion that IL-6 blockade may yield beneficial effects in RA. IL-6 knockout mice are resistant to CIA and show reduced levels of serum TNF-α (210). IL-6 signals through the IL-6R (IL-6Rα and gp130 sub-units) inducing the secretion of acute phase proteins (CRP and serum amyloid A), inflammatory chemokines (IL-8 and MCP-1) and up-regulates the expression of adhesion proteins (211). IL-6 signalling induces the accumulation of monocytes, promotes neutrophil apoptosis and macrophage phagocytosis akin to the resolution phase of many inflammatory reactions and which may reflect some of the pleiotropic effects attributable to IL-6 (211). To date blockade of IL-6 signalling has been successfully achieved in clinical trials using an antibody generated against the IL-6R to inhibit IL-6 binding. Treatment with Tocilizumab, a humanised anti-human IL-6R monoclonal antibody, in RA patients with active disease revealed an improvement in all disease activity assessments in a dose responsive manner; normalisation of CRP levels and a significant improvement in RF titres relative to the placebo group (212). IL-6 blockade is therefore an attractive therapeutic target and the development of soluble IL-6 receptors, similar to anti-TNFα therapy, are being investigated (213, 214).

6.3.5.5 Interleukin-1 superfamily

The IL-1 family comprises eleven structurally related cytokines of which the biological activity of only five has been well characterised, namely IL-1α, IL-1ß, IL-1 receptor antagonist (IL-1Ra), IL-18 and IL-33. Early in vivo studies demonstrated that injection of IL-1 into the knee joint of rats caused symptoms akin to chronic arthritis due to the secretion of matrix metalloproteinases (MMPs) and proteoglycans mediated via macrophage activation, causing cartilage and bone destruction. RA is associated with elevated levels of IL-1 in the

synovium. Thus much effort has been directed towards finding appropriate methods of inhibiting IL-1 activity. Despite positive pre-clinical studies, IL-1 blockade using Anakinra (IL-1 receptor antagonist) has failed to provide adequate therapeutic value in a large enough number of patients. It is not clear whether this reflects disease heterogeneity, suboptimal drug design (e.g. short half life) or an inadequate understanding of the role of IL-1 in the RA synovial cytokine hierarchy. Studies using anti-TNF in combination with IL-1Ra provide an additive functional effect upon cytokine inhibition from synovial tissue *in vitro* (215).

IL-18 is synthesised as a 23kD pro-molecule that is cleaved by caspase 1, proteinase 3 or elastase to an 18kD ligand which is then able to bind IL-18R. The level of IL-18 within RA serum and synovial fluid correlates with DAS28 and CRP and suppression of disease by DMARD therapy (165, 216). IL-18 is able to induce expression of multiple cytokines and chemokines, particularly TNFα, in both *in vitro* and *in vivo* models whilst promote the differentiation of $CD4^+$ T cells towards a Th1 phenotype in the presence of IL-12. IL-18 has been also shown to induce vascular endothelial growth factor (VEGF) expression in fibroblast like synoviocytes implicating IL-18 in angiogenesis. Multiple approaches have therefore been employed in an attempt to inhibit its pro-inflammatory effects (217). Administration of anti IL-18 antibody suppressed streptococcal cell wall arthritis (218). Similar to IL-1 it is unclear what proportion of RA pathology is attributable to the effects of IL-18 and the efficacy of IL-18 targeted therapy in RA remains to be identified.

There is no doubt that cytokines represent valid therapeutic targets for the treatment of RA, exemplified by the success of anti-TNFα therapy. However, it remains unclear to what extent distinct patterns of synovial cytokine hierarchy exist within clinical subsets of RA. This is therefore a source of uncertainty in the ability to predict clinical effectiveness of specific cytokine blockade alone. However, the combination of cytokine blocking agents may prove more efficacious in the treatment of RA. A summary of additional relevant cytokine activities is included in Table 6.3.

Cytokine	Cellular source	Role in rheumatoid arthritis
IL-1	Monocytes, B cells & fibroblasts	Induces cytokine secretion from fibroblasts and monocytes and activates osteoclasts.
IL-6	Monocytes/ macrophages, fibroblasts, lymphocytes	Induces proliferation of lymphocytes and differentiation of Th17 cells. Supports B cell antibody production.
IL-12	Macrophages & dendritic cells	Primarily secreted upon exposure to TLR ligands and induced maturation and proliferation of Th1 cells.
IL-15	Monocytes, fibroblasts, mast cells, neutrophils & dendritic cells	Promotes activation of T cells, fibroblasts, macrophages and neutrophils.
IL-17	T cells, macrophages, dendritic cells & γδ T cells	Induces the secretion of pro-inflammatory cytokines from macrophages, fibroblasts and endothelial cells.
IL-18	Monocytes, neutrophils & dendritic cells	Promotes differentiation of T cells to a Th1 phenotype and induces activation of monocytes/ macrophages.
IL-23	Macrophages & dendritic cells	Secreted in response to TLR ligands and promotes survival and expansion of Th17 cells.
APRIL	Monocytes/ macrophages & lymphocytes	Member of the TNF superfamily and promotes B and T cell survival and proliferation.

BAFF	T cells, monocytes/ macrophages & dendritic cells	Member of the TNF superfamily and promotes B cell survival and maturation.
GM-CSF	Monocytes, osteoclasts, fibroblasts & T cells	Stimulates the differentiation and survival of neutrophils and macrophages.
MCP-1	Osteoclasts and macrophages	Exert chemotactic effects upon macrophages, dendritic cells, neutrophils and lymphocytes.
M-CSF	Lymphocytes, monocytes, fibroblasts and osteoclasts	Regulator of monocyte differentiation and proliferation and macrophage survival.
MIP-1α/β	Macrophages	Exert chemotactic effects upon macrophages, dendritic cells, neutrophils and lymphocytes.
TNFα	Adipocytes, monocytes/ macrophages, fibroblasts, T cells and B cells	Induces the activation of macrophages and endothelial cells whilst promoting apoptosis of neutrophils. Inhibits the regulatory function of regulatory T cells.

Table 6.3 Cytokines and chemokines implicated in the pathology of rheumatoid arthritis
A brief description of the cellular source and the role of the major cytokines and chemokines implicated in the development of rheumatoid arthritis. Interleukin (IL), a proliferating inducing ligand (APRIL), B cell activating factor belonging to the TNF family (BAFF), granulocyte/ macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant protein -1 (MCP-1), macrophage colony stimulating factor (M-CSF), macrophage inflammatory protein (MIP) and tumour necrosis factor α (TNFα).

6.3.6 Animal models of rheumatoid arthritis

The complex cellular and cytokine mediated interactions requires sophisticated modelling capabilities. Whereas these may be achieved *in vitro* it is not always possible to recapitulate the *in vivo* inflammatory environment. Furthermore, clinical studies analysing the potential pathological components of RA are often limited by a restricted time window of observation and, along with genetic and /

or environmental diversity, means that interpretation of results can often be difficult. It is therefore not uncommon to find contradictory observations generated from different clinical studies; e.g. it is still not clear whether lipids, particularly total cholesterol, is increased, decreased or stays the same and when these changes may occur in the development of human RA. Therefore, animal models have been used extensively in studies of RA pathogenesis to allow dissection of pathways involved in the disease and despite their limitations, have significantly contributed towards major advances in the development of novel therapeutics. This is exemplified in the development of anti-TNFα therapy in the murine (CIA) model (199). However it is important to realise the limitations and advantages of commonly used animal models of arthritis and their comparison to the human disease. There are many rodent models of arthritis although many of them are not as directly comparable to the pathology of human RA as CIA. The aspects of CIA are described below. However, whilst many other models have been used to study the arthritis e.g. collagen antibody induced arthritis, zymosan arthritis and the human TNFα transgenic mouse, these will not be discussed here but have recently been reviewed by Asquith *et al* (219) and are summarised in Table 6.4.

6.3.6.1 Collagen-induced arthritis

Collagen-induced arthritis shares many similarities with human RA. In particular, breach of tolerance and generation of auto-antibodies towards self collagen are generated, and as such CIA is considered the gold standard *in vivo* model to study RA. CIA was first described in rats and subsequently shown to be inducible in susceptible strains of mice, following inoculation with type II heterologous collagen in Complete Freund's Adjuvant (CFA) (220, 221). Susceptibility is restricted to strains that have MHC Class II I-Aq and I-Ar haplotypes, e.g. DBA/1, similar to RA in humans (222). CIA can also be initiated in non-human primates, making it a useful model in which to better assess efficacy of novel therapeutic targets and aid their transition through the primary stages of pre-clinical development. Male mice are most widely used to exclude complications with sex hormones although CIA is not considered to be sex biased. Clinical signs of disease typically develop 21 to 25 days after the initial inoculation and presents as a polyarthritis which is most prominent in the limbs and characterized by synovial inflammatory infiltration, cartilage and bone erosion and synovial

hyperplasia similar to human RA. Disease severity is expected to peak at approximately day 35 after which DBA/1 mice enter remission, marked by increased concentrations of serum IL-10 and a subsequent decrease in pro-inflammatory Th1 cytokines (223). In a development of the model, inoculation with homologous type II collagen has been reported to cause chronic relapsing arthritis more akin to human RA which has been suggested to be more useful for studying remission inducing therapies (224).

The ability to study the effect of genetic modification or targeted gene deletion has been problematic in the DBA/1 model as most transgenic mice are on the C57BL/6 background which is generally considered resistant to CIA. However, with the recent development of a refined protocol for the induction of CIA in C57BL/6 mice, the possibility to study arthritis in genetically modified mice is now more amenable (225, 226). There are differences in the onset and progression of disease between the DBA/1 and C57BL/6 which may cause discrepancies when comparing studies between these two strains. The C57BL/6 strain develops arthritis approximately 4 to 7 days later eventually reaching severity at a level comparable to arthritis in DBA/1 mice but which is sustained as opposed to the remitting / relapsing arthritis observed in DBA/1 mice (224). This was associated with sustained levels of serum anti-collagen antibody titres, higher levels of T cell proliferation and IFNγ secretion in the late stage of disease. Therefore, whilst both strains of mice may be a useful to study the preclinical development or prophylactic treatment of arthritis, the C57BL/6 mice may be more suited for analysis of late / established arthritis.

As eluded to above the CIA model of arthritis is a widely used and is therefore a very well characterised model of arthritis. This is may be in part through the relative ease as to which arthritis can be induced with the CIA protocol unlike the antigen and zymosan induced arthritis which requires a high level of operator skill to be able to accurately perform intra-articular injections in mice. The CIA model has several more advantages over other *in vivo* models in which to study potential interventions in RA. CIA model is a polyarthritis affecting any and potentially all of the limbs and by analysis of serum cytokines it is evident that there is a systemic elevation of multiple pro-inflammatory cytokines and chemokines akin to human disease. This is in comparison to the zymosan model of arthritis which is monoarthritic and may therefore not reflect the systemic

nature of disease. Additionally, the CIA model involves both the innate and adaptive arms of the immune response whereas inflammation in the collagen antibody-induced arthritis relies upon complement activation and is therefore predominantly mediated through macrophage and polymorphonuclear cell activation. Although useful for dissecting out a role for particular arms of the immune response, once again this does not reflect the inflammation in RA. Finally, with all interventions in which the aim is to inhibit disease progression drugs are required to trialled in primates prior to clinical trials. With the ability to induce arthritis in primates as well as rodents the CIA model is ideal for the development of potential therapeutics. Therefore, the CIA model was selected for all future studies to test whether LXR agonists were potential therapeutics for the treatment of RA.

Model	Species	Characteristics	Ref.
Collagen-induced arthritis	Mouse, Rat, Rabbit, non-human primate	Inducible in susceptible strains of rodents Polyarthritis associated with antibody and T cell responses. Low incidence and variability of disease severity in C57BL/6 mice. Inoculation with homologous collagen induces relapsing/ remitting arthritis but otherwise is self limiting.	(220, 221, 225, 227, 228)
Collagen antibody-induced arthritis	Mouse,	Self limiting polyarthritis in 100% animals but limited to macrophage and polymorphonuclear cell involvement. Can be induced in most strains of mice.	(182, 183, 229)
Zymosan-induced arthritis	Mouse, Rat	Monoarthritis and requires a high degree of technical ability but can be induced in multiple strains of mice. TLR 2 dependent and therefore recapitulates infection driven pathology.	(230, 231)
Antigen induced arthritis	Mouse, Rat	Requires a high degree of technical ability and precludes analysis of the systemic component of disease.	(232, 233)
Spontaneous transgenic models of arthritis.	Mouse	Spontaneous chronic and progressive polyarthritis, onset of disease at 3-4 weeks of age. This includes the KBxN, SKG and DNase II-/-IFN-IR-/- and human TNFα transgenic mice. These mutations have so far only been identified in mice.	(200, 234-236)

Table 6.4 Animal models of arthritis.
The characteristics of the most commonly used animal models of arthritis with explanation of their advantages, limitations and the species they can be induced in.

6.3.7 Cardiovascular disease in rheumatoid arthritis

RA is associated with multiple co-morbidities and reduced life expectancy. The increased mortality rate is largely attributable to cardiovascular disease (CVD) and many studies have now suggested that CVD contributes directly to disease pathology emphasising the systemic nature of RA. I shall give detailed consideration to this co-morbidity since one attraction of the LXRs in RA as a regulatory molecular pathway resides in their capacity to modulate both inflammatory and metabolic events of relevance.

6.3.7.1 Atherosclerosis

Atherosclerosis is characterised by the formation of fatty lesions (plaques) in arterial vessel walls. The environment within an atherosclerotic plaque is highly pro-inflammatory and plaque progression is associated with immune cell infiltration, lipid accumulation and the secretion of multiple pro-inflammatory cytokines and chemokines. Formation of an atherosclerotic plaque is suggested to be initiated by vascular endothelial cell damage and subsequent activation, although the precise mechanisms that induce damage are not known. Hypercholesterolemia is a major risk factor for the development of atherosclerosis and predicts disease progression. Indeed, feeding mice an atherogenic diet correlates with plaque size development (237). Low density lipoprotein (LDL) cholesterol can penetrate the vascular endothelium into the intima where it can be oxidised forming oxidised LDL (oxLDL) causing endothelial cell activation in which the expression of the adhesion molecules, typically VCAM-1 and ICAM-1, are up-regulated (238). In addition, to an elevated plasma lipid profile, hypertension is another well recognised risk factor for the development of atherosclerosis. Atherosclerotic plaques generally form in arteries and are typically located at sites of hemodynamic strain; high blood flow and shear stress. Exposure of human umbilical vein endothelial cells (HUVECs) to dynamic flow *in vitro* induces cytoskeleton rearrangements, NF-κB activation and the expression of VCAM-1, ICAM-1 and IL-1β (239). Furthermore, hemodynamic stress may also enhance the “leakiness” of the vascular endothelium allowing LDL penetration into the intima and thereby present a mechanism by which hypercholesterolemia and hypertension may synergise to promote vascular inflammation.

Inflammatory cells, typically monocytes and T cells, roll and bind to the adhesion molecules expressed on the blood vessel wall and then migrate into the intima directed by the interactions of chemokines and chemokine receptors e.g. MCP-1/ CCR2 and Rantes/ CCR1. Differentiation of monocytes into macrophages is necessary for the progression of atherosclerosis demonstrated by the amelioration of plaques in M-CSF$^{-/-}$ mice (240, 241). Macrophage differentiation is marked by the up-regulation of scavenger receptors which internalise a variety of lipoprotein molecules including oxLDL (242). In a state of hypercholesterolemia the rate of oxLDL uptake exceeds the rate of cholesterol clearance, excess cholesterol is therefore stored in cytoplasmic lipid droplets resulting in macrophage foam cell formation. Lipid loading of macrophages is also associated with the secretion of IL-1β, TNFα, MMPs and augmentation of oxidative stress (243, 244). Accumulation of foam cells and extracellular lipids forms a lipid core within a plaque which is surrounded by a cap of endothelial cells, smooth muscle cells and a collagen matrix. Atherosclerotic plaques are generally stable structures which can occlude the vessel lumen reducing blood flow and may cause angina or tissue ischemia but in a large proportion of patients the presence of atherosclerotic plaques are asymptomatic. However, plaque destabilisation can cause plaque rupture and thrombus formation leading to myocardial infarction and/ or stroke. Plaque destabilisation can occur through a variety of mechanisms including the actions of pro-inflammatory cytokines and proteases as found in RA (245-247). It is therefore evident that inflammation is central to plaque development. Understanding the inflammatory processes and how they interact with lipid metabolism is now subject to intense investigations for the development of future therapeutic targets for the treatment of atherosclerosis.

6.3.7.2 Cardiovascular disease related mortality in rheumatoid arthritis

The life expectancy of patients with RA is significantly reduced by up to 10 years and death is predominantly due to cardiovascular disease (CVD) related events. One study followed 603 RA patients relative to 603 sex and age matched healthy controls from the same geographical population for the development of coronary heart disease (CHD) related events (248). Data regarding CHD events was collected retrospectively from the subjects' medical records over a mean period of approximately 15 years. This study revealed that the risk of myocardial

infarctions in the RA population was more than five times more prevalent than that in the non-RA population. Similarly, the number of sudden deaths was twice that found in the non-RA population and in total nearly 50% of the RA subjects died of CVD related symptoms. These results were confirmed by an independent study which prospectively followed 114,342 women for the development of RA and CVD (134). The study revealed that of the 527 women that developed RA the prevalence of myocardial infarctions was approximately three fold higher than compared to subjects without RA. Although in both studies cigarette smoking was significantly more frequent in the RA cohorts the observations that CHD is increased in RA subjects could not be explained by other traditional CVD risk factors e.g. BMI, type II diabetes and hypertension. This therefore suggests that RA itself may be a risk factor for the initiation and progression CVD. Indeed a follow-up study revealed that Erythrocyte Sedimentation Rate (ESR), RF seropositivity, joint swelling and radiographic changes, as markers of systemic inflammation and RA disease severity, conferred a significant additional risk for CVD related deaths in subjects with RA (249). Interestingly the duration of RA disease had no impact upon CVD related outcomes. Subsequently many studies have tried to investigate the specific aspects of RA associated CVD with particular emphasis on potential changes in vascular function and alterations in serum lipids.

6.3.7.3 The atherogenic profile in rheumatoid arthritis

It is evident that prevalence of CVD and associated risk factors are elevated in RA. Indeed, carotid intima media thickness (IMT), as an indicator of atherosclerotic plaques, is increased in RA (250, 251). Many studies have since confirmed these observations and carotid IMT has been shown to predict the development of CVD in RA (252). Furthermore, it is evident that there are changes in the plasma lipid profile up to ten years prior to the clinical onset of RA (253). This was marked by an increase in plasma triglycerides and total cholesterol concentrations but a decrease in protective HDL-cholesterol. Although altered levels of serum lipids were suggested to be a potential mediator for the manifestation of clinical RA RF is also detectable many years prior to the clinical development of RA. This supports the consensus that sub-clinical RA is present many years prior to its clinical manifestation. However whether cardiovascular disease drives the manifestation of clinical RA remains

unknown. Similarly, analyses of lipid profiles in patients with established RA have demonstrated increased concentrations of triglycerides and in some cases total cholesterol but a decrease in HDL-cholesterol which negatively correlated with CRP (254, 255). These studies suggest that the lipid profile in RA is pro-atherogenic and may accelerate the formation of atherosclerotic plaques. Moreover, it is plausible that the inflammatory profile associated with RA may drive plaque destabilisation and may be in part responsible for the increased prevalence of myocardial infarctions and strokes in RA.

It is now widely accepted that the inflammatory state associated with RA may promote atherogenesis however, how dyslipidemia and the inflammatory aspect of atherosclerosis impacts synovial inflammation is not known. However, oxLDL has been detected in RA synovial fluid and serum oxLDL concentrations correlate with CRP (256, 257). Elevated levels of oxLDL may promote macrophage activation directly within the synovium or indirectly by the secretion of pro-inflammatory cytokines from atherosclerotic plaques.

A better understanding of how inflammation and metabolism interact to drive the pathology of RA and associated CVD are therefore required for the development of therapeutics with higher degree of efficacy. Given the multifactorial nature of RA modern therapeutics may have to be more than just modulators of inflammation and treating RA associated co-morbidities may improve disease prognosis. Indeed it is already evident that a reduction of general inflammation in RA by some therapeutics is associated with an improved lipid profile as discussed below.

6.3.8 Current therapeutics

With an advancing knowledge of the mechanisms that drive RA pathogenesis the expectations of modern therapeutic regimes are now much greater. It is now no longer the aim to simply inhibit disease progression (although this reflects current clinical reality) but rather prevent the initial onset of disease through the identification of novel therapeutic targets and better biomarkers of disease activity.

6.3.8.1 DMARDs

Disease modifying anti-rheumatic drug (DMARDs), in particular Methotrexate, have been widely used for the treatment of RA for nearly three decades. Several studies demonstrated that Methotrexate use is associated with improvement in disease activity e.g. measured as DAS28 in a proportion of patients (258-260). However, clinical improvements were often of short duration and in general a 70% improvement of disease severity, ACR70 response, was only observed in less than 15% of patients (261). Even in the patients that enter clinical remission (DAS28 $\leq$ 2.6) radiographic bone erosion is irreversible. Several studies suggest that radiographic damage progresses in some patients receiving DMARD therapies despite exhibiting 'satisfactory' clinical responses (262-264). Furthermore, up to 60% of patients have reported adverse reactions to Methotrexate including nausea, diarrhoea, hepatitis and leukopenia which have only been resolved upon discontinuation of therapy (260). Methotrexate therefore does not exert therapeutic benefits in a large enough proportion of patients and simply delays the progression of disease onset and severity. Other DMARDs include sulphasalazine, hydroxychlorquine, leflunamide, and gold all of which mediate clinical benefit in a proportion of patients but often associated with significant toxicity and limited duration of benefit. Crucially none are used on the basis of sound pathological understanding of RA for which mechanism specific drug selections have been made. Combinations of these agents have been shown to increase the benefits achieved and paradoxically to reduce adverse events. Despite this remission rates remain low and drug retention over the course of a chronic illness remains unacceptably low.

Glucocorticoids have been widely used as an anti-inflammatory therapeutic in RA with some success. Glucocorticoids predominantly mediate their effects in RA via the suppression of T cell proliferation but also promote apoptosis of neutrophils whilst inducing macrophage phagocytosis and thereby promote inflammatory resolution (265-267). However, although glucocorticoids exert predominantly anti-inflammatory effects it is now well recognised that glucocorticoids are implicated in the progression of various metabolic disorders (268-270). Indeed, corticosteroid therapy was associated with a two fold increase of CVD related deaths in RA (248, 249, 271). The relationship between inflammation and cardiovascular disease in RA is complex. However, with an

increased understanding of the mechanisms that drive RA pathogenesis several novel biologics have been developed that target either the cellular source(s) or the components of molecular pathways that mediate inflammation. As such modern biologics have given promise of more efficacious therapies for the treatment of RA.

6.3.8.2 Rituximab and B cell targeted therapies

Rituximab is a humanised monoclonal antibody which targets the cell surface protein CD20 exclusively expressed on mature B cells, but not plasma cells. The use of rituximab as a potential immuno-therapy was first demonstrated in primates where it was shown to potently induce B cell depletion (272). Rituximab was first used as a treatment for non-Hodgkin's lymphoma but was subsequently shown to be a highly effective therapy in RA (273, 274). Although several studies have suggested that CD20 may function as a calcium channel or as a cell surface signalling receptor the function of CD20 and the mechanism by which rituximab initiated B cell depletion in RA remains unknown (275). Several mechanisms have been suggested including antibody dependent cell mediated cytotoxicity, complement dependent cytotoxicity and inhibition of NF-κB. Effects in RA synovitis may result from reduced antibody production, altered antigen presentation or reduced secretion of B cell derived pro-inflammatory cytokines. Beneficial effects of single doses of rituximab have been reported from 3-12 months whilst repeated courses of therapy have lead to improvements lasting up to 5 years thus far (276). Interestingly, Rituximab was shown to transiently improve endothelial dysfunction (277). As such B cell depletion is now a favourable strategy for the treatment of RA. Therefore much effort has been expended in developing biologics against BLyS (B-lymphocyte stimulator) and APRIL (A Proliferation Induced Ligand) which are members of the TNF superfamily of cytokines and promote B cell survival, differentiation and activation. Belimumab an anti-BLyS human monoclonal antibody is now in clinical trials for the treatment of systemic lupus erythematosus (SLE) and similar studies are likely to be extended to RA (278).

6.3.8.3 Anti-TNFα

TNFα blockade in the clinic is clearly associated with a reduction in articular inflammation and damage (279, 280). There are now three TNFα antagonists, Infliximab, Etanercept and Adulimumab, which are used for the treatment of RA with varying modes of TNFα inhibition. Two further agents will shortly be generally available namely golimumab and certolizumab. Infliximab and Adalimumab are humanised monoclonal antibodies specifically raised against TNFα whereas Etanercept is a TNF-receptor Fc fusion protein and acts as a soluble receptor. In all cases TNFα is prevented from interacting with the TNF receptor on the surface of immune cells. Although, TNFα blockade is more effective than methotrexate alone it is effective in approximately 70% of patients and is usually used in combination with MTX with which it has synergistic benefits (281). In addition to the anti-inflammatory effects treatment with anti-TNFα therapeutics has been shown to reduce carotid intima-media thickness and improve the atherogenic lipid profile in RA, although this remains controversial (282-284). Once again therefore novel biologics suggest a relationship between inflammatory pathways and vascular events in the RA population.

6.3.8.4 Anti IL- 6 Receptor

The success of TNFα blockade has raised the expectations for novel therapeutics and provided proof of concept that cytokine blockade represents a valid therapy in RA. However, as anti-TNFα therapy is not effective in a proportion of patients other therapeutic options are required. In particular anti-IL-6R therapy with Tocilizumab, a humanised monoclonal antibody against the IL-6 receptor has provided encouraging results in patients who have previously failed anti-TNFα therapy (212, 285, 286). However, moderate increases in serum total cholesterol and triglyceride concentrations were observed (212). Similar studies are likely to be extended to other cytokines with clinical trials already proceeding for IL-17 and IL-12/23 neutralising therapies and targeting GM-CSFR (168, 287, 288).

6.3.8.5 Abatacept

Abatacept is a fusion protein of the extracellular domain of the cytotoxic T lymphocyte-associated antigen 4 (CTLA4) linked to the Fc portion of human IgG1. Abatacept functions to inhibit the activation of T cells by blocking co-stimulation through the binding of CTLA4 to CD80/86 expressed on the plasma membrane of antigen presenting cells. During clinical trials treatment of RA patients with abatacept yielded a high ACR response and is now currently approved in Europe for the treatment of RA (289).

6.3.8.6 Statins

As cardiovascular risk is associated with increased morbidity and mortality statins were trialled to assess their therapeutic potential in RA. Statins inhibit 3-hydroxy3methylglutarylcoenzyme A reductase (HMG-CoA) which is the rate limiting enzyme in the synthesis of cholesterol and are therefore widely used for the treatment of atherosclerosis. Treatment of patients with Atorvastatin was shown to modestly inhibit disease severity in RA associated with a decrease in ESR and CRP and improved measures of vascular function and atherogenic lipid profiles (290, 291). Whilst at first it was not clear if these beneficial effects were attributable to lipid lowering mechanisms or direct regulatory effects upon inflammation simvastatin has since been shown to inhibit cytokine secretion from synovial fibroblasts (292, 293). These studies demonstrate that statins may be able to both improve the atherogenic lipid profile and exert direct anti-inflammatory effects providing support of statins as a therapy in RA which are predicted to improve morbidity whilst reducing CVD associated mortality. Certainly many studies indicate that statins mediate anti-inflammatory effects in vitro and therefore the foregoing is plausible.

7 Materials & Methods

7.1 General reagents & buffers

7.1.1 Materials and reagents

General chemicals: All chemicals were purchased from Sigma (UK) unless otherwise indicated.

Plastics: All plastics used for cell culture were purchased from Corning and Gibco unless otherwise indicated.

Avertin®: 3g 1,1,1 tribromoethanol was mixed with 3ml amyl alcohol and placed on a rotator to fully dissolve. Avertin should be stored at 4^{o}C in the dark. Stock Avertin was diluted 1:40 with PBS before use and given at a dose of 250 mg/kg (~500 µl).

Lipopolysaccharide (LPS): Ultra pure LPS (Calbiochem) derived from *Escherichia coli* was dissolved in sterile water at a stock concentration of 1 mg/ml and used at a final concentration of 100 ng/ml.

LXR agonists: GW3965 and T0901317 (T1317) were synthesised by Schering-Plough Corporation (UK) and supplied in 2 g vials. Both T1317 and GW3965 were dissolved in 5% Mulgofen (GAF Co, UK)/ PBS or 5% Chremophore (Sigma)/ PBS at the indicated dose and administered daily *in vivo* by either intraperitoneal injection, sub-cutaneous injection or by oral gavage as indicated. The endogenous LXR agonists 24(S), 25-epoxycholesterol, 22(S) hydroxycholesterol and 22(R) hydroxycholesterol were purchased from Biomol or Sigma. For *in vitro* use all agonists were dissolved in DMSO (Riedel-de Haen).

7.1.2 Buffers & culture media

0.5M EDTA pH8 (1L): 186.1 g ethylenediaminetetraacetic acid (EDTA) and 20 g NaOH was mixed with 800 ml dH_2O adjusted to pH8 and then made up to 1 L with dH_20.

0.1 M Bicarbonate buffer (50 ml): 0.42 g sodium bicarbonate ($NaHCO_3$) was dissolved in a final volume of 50 ml dH_2O and stored at 4^{o}C.

Complete media: RPMI1640, 10% heat inactivated foetal bovine calf serum, Penicillin (100 units/ml), Streptomycin (100 µg/ml) and L-Glutamine (2 mM) (all at final concentration from Invitrogen).

Decalcificaiton buffer: 5.5 g of EDTA disodium salt was dissolved in 90 ml dH_2O and made up to a final volume of 100 ml with formaldehyde.

ELISA wash buffer: 1X PBS was mixed with 0.05% (final concentration) Tween 20.

PBS: Phosphate buffered saline was purchased from Invitrogen.

PEA (PBS, EDTA, ALBA): 500 ml PBS, 1 ml 0.5 M filter sterilised EDTA pH8 and 2.5 ml 4.5% ALBA (Bio Products Laboratory).

Tris-acetate-EDTA (TAE) buffer: To make a 50x stock solution of TAE, 242 g of Tris base was dissolved in 750 ml dH_2O. This was then mixed with 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (Ph8). The final volume was then made up to 1000 ml with dH_2O. The buffer was then diluted 1:50 before use with dH_2O.

Trypan blue: 0.4 g Trypan blue was mixed with 80 ml PBS and brought to a slow boil, cooled to room temperature and made up to 100 ml with PBS. Stock Trypan blue was then diluted 1:2 with PBS before use.

Tail tip lysis buffer: 1 ml 2 M Tris, 20 ml 0.5 M EDTA, 6.7 ml 3 M NaCl and 10 ml 10% Sodium Dodecyl Sulphate (SDS) was dissolved in a final volume of 200 ml dH_20. 500 µg/ml proteinase K (20 mg/ml stock concentration, Qiagen) was then added fresh before use.

7.2 In vivo procedures

7.2.1 Animal welfare

All animals were housed in pathogen free conditions within the Biological Services Joint Research Facility (JRF) at the University of Glasgow. Animals had access to food and water *ad libitum*. All procedures were carried out in

accordance with project licences approved by the United Kingdom Home Office and in accordance with the Animals (Scientific Procedures) Act 1986.

7.2.2 Mice

C57BL/6 or DBA/1 mice: Male mice were purchased from Harlan at approximately eight weeks of age and allowed to acclimatise for one week before experimental procedures.

LXR null mice: LXRα knockout (KO) or LXRß KO mice and wild-type (WT) littermates on a C57BL/6 background were generated by Lexicon but supplied by Schering Plough Corporation (UK). These were bred in house and used to generate LXRα/ß double KO mice (See results).

All mice were culled by a recommended schedule 1 method or terminal anaesthesia by intraperitoneal injection of 700 µl of Avertin.

7.2.3 Induction of Collagen-induced arthritis

7.2.3.1 Induction of CIA in DBA1 mice.

Induction of collagen-induced arthritis (CIA) was firstly described in rats (221) and subsequently in susceptible strains of mice (220). Male DBA/1 mice at 8 weeks of age were used for induction of collagen-induced arthritis. 2 mg/ml type II bovine collagen (MD Biosciences) dissolved in 0.05 M glacial acetic acid was thawed overnight at 4^{o}C. On the day of immunisation the collagen was mixed with an equal volume of 4 mg/ml Complete Freund's Adjuvant (CFA – MD Biosciences) from M. Tuberculosis, to yield a final concentration of 1 mg/ml collagen. The collagen/ CFA mixture was emulsified on ice using a hand held homogeniser. To check the stability of the emulsification, 1 drop of the emulsion was dropped into a beaker of water. The emulsion was considered to be stable if it remained as a solid on the water surface.

On the day of immunisation (day 0) the tail base of each mouse was shaved and the skin sterilised with 70% ethanol. Each mouse was intradermally injected with 50 µl of the collagen/ CFA emulsion either side of the tail base so that each

mouse received a total of 100 µg of collagen (Figure 7.1). Mice were monitored daily for any signs of ulceration.

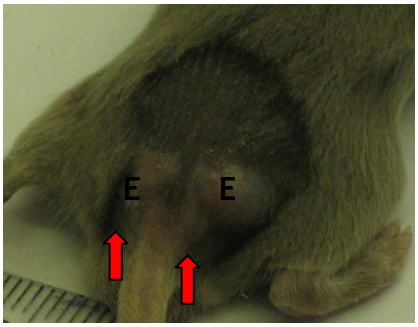


Figure 7.1 Injeciton sites for induction of collagen induced arthritis.
The collagen/ CFA is injected intradermally; the needle is inserted either side of the tail base (indicated by the arrows) and then pushed in approximately 1 cm further in where the emulsification was injected (E).

Three weeks post immunisation (day 21) 2 mg/ml bovine type II collagen was mixed with an equal volume of PBS. Each mouse was injected intraperitoneally with 200 µl (200 µg) collagen/ PBS. Mice were monitored daily for signs of disease.

7.2.3.2 Induction of CIA in C57BL/6 mice

C57BL/6 mice are generally considered resistant to the induction of arthritis as they have the H-2b haplotype. Induction of CIA in wild-type or transgenic mice on C57BL/6 background (8 – 12 weeks old) was carried out following the protocol described by Inglis JJ *et al* (225). Type II chicken collagen dissolved in 0.05 M glacial acetic acid (2 mg/ml - MD Biosciences) was mixed with an equal volume of 4 mg/ml CFA and emulsified using a hand held homogeniser and the stability of the emulsification checked as above.

On the day of immunisation (day 0) the tail base of each mouse was shaved and the skin sterilised with 70% ethanol. Each mouse was injected intradermally with 50 µl of the collagen/ CFA emulsification at two sites either side of the tail base (total of four injections) so that each mouse received a total of 200 µg of collagen. Mice were monitored daily for any signs of ulceration.

Three weeks post immunisation (day 21) 2 mg/ml chicken type II collagen was mixed with an equal volume of PBS. Each mouse was injected intraperitoneally

with 200 µl (200 µg) collagen/ PBS. Mice were monitored daily for signs of disease.

7.2.4 Clinical assessment of arthritis

Mice were monitored for clinical signs of disease from day 21 onwards. Clinical signs were assessed by micro-calliper measurements which measured any swelling of the wrist joint. Secondly, a clinical score was assigned to each paw based upon disease severity where 1 = swollen digit(s), 2 = erythema, 3 = swollen paw/ ankle and 4 = loss of function, visualised and described in Table 7.1, allowing a maximum score of 16 per animal.

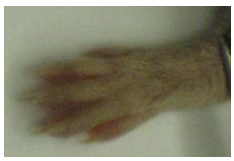
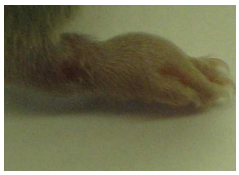
Score	Description	Clinical appearance	Comments
0	No disease		No swelling or redness of any digits or any part of the lower leg.
1	Swollen digits		Swelling of individual or multiple digits, sausage like in appearance, but not extending into the upper region of the paw.
2	Erythema		Redness of the paw. This was usually transient and lead to swelling of the paw.
3	Swollen paw/ ankle		Swelling of paw and/ or ankle extending from the toes along the length of the paw.
4	Loss of function		Usually accompanied by even more swelling. Function of front paws was assessed by the ability to grip a cage lid and/or for the rear paws the ability to walk up a cage lid held at an inclination of ~45°.

Table 7.1 Visualisation of murine arthritis.
Development of arthritis was assessed by clinical observations in which a maximum score of 4 was assigned to each paw (maximum score per animal was 16).

7.2.5 Histological assessment of arthritis

For histological assessment rear paws were prepared by fixation for three weeks in 10% neutral buffered formalin (Sigma) and subsequent decalcification for three weeks in decalcification buffer, which was changed weekly, as described

(294). Paws were cut to 7 µM sections and stained with haemotoxylin and eosin (H&E, See below for details). The severity of arthritis was assessed by a blind observer and a histological score assigned. The severity of inflammatory infiltrate was scored as 0 = normal, 1 = minimal inflammatory infiltrate, 2 = synovial thickening and infiltration, 3 = moderate thickening and 4 = severe thickening more than width of epiphysis. Articular destruction was separately graded as 0 = normal, 1 = erosion of $\leq$ 25% of articular surface, 2 = 26-50%, 3 = 51- 75% and 4 = $\geq$ 75%.

7.3 Ex vivo procedures

7.3.1 Preparation of paws for histological analysis

7.3.1.1 Tissue processing, embedding & sectioning

Tissue processing

Paws were removed from mice by cutting through the leg just above the ankle joint and the toes removed to aid the diffusion of buffers into the tissue. The paws were placed in approximately 5 ml of neutral buffered formalin (Sigma) for 2 weeks at room temperature. After fixation the paws were then decalcified by incubating the paws in decalcification buffer (see buffers above) on a rotator for two weeks at room temperature. During this period the buffer was replaced approximately every three days to prevent re-deposition of calcium.

The tissues were then dehydrated in an automated tissue processor (Thermo, Citodel 1000) which immersed the tissues into increasing concentrations of ethanol until they were completely dehyradated and then immersed in wax, to allow diffusion into the tissue; detailed below:

1. Neutral buffered formalin 30 min
2. 70% ethanol 1 hrs
3. 90% ethanol 1 hrs

4. 95% ethanol 1 hrs
5. 100% ethanol 2 hrs X 3
6. Xylene 2 hrs X 3
7. Paraffin wax 4 hrs X 2

Tissue embedding

Once the samples were processed they were embedded into wax blocks (Thermo histocentre 3) and allowed to cool. The blocks were then stored at 4^{o}C until they were required for sectioning.

Sectioning of tissue

Firstly, the blocks were pared on the microtome set at 25 µm to allow quicker cutting through the wax. Once the tissue was reached the blocks were removed and placed on ice cold water to harden the paraffin wax. Once cooled the microtome was set to cut at a thickness of 7 µm and the required number of sections cut in ribbons. The ribbons of wax/ tissue were then placed in a floating water bath set at 40^{o}C. Each section was then separated using a scalpel and then placed on a charged frosted microscope slide (Superfrost plus, VWR) and placed on a hotplate (Raymond A Lamb Hotplate) at 55^{o}C at allowed to dry for approximately 30 min. The slides were then stored at 4^{o}C until needed for staining.

7.3.1.2 Haematoxylin and Eosin staining

Paws were embedded and prepared in paraffin sections as described above. The sections were then stained with Haematoxylin and Eosin (H&E) as described below. All reagents were purchased from Sigma. The ethanol solutions were prepared by diluting 100% ethanol with dH_2O to the required final percentage (v/v).

Firstly, the wax was softened by incubating the sections in an oven (GenLab) at 60^{o}C for approximately 35 min and then the wax removed from the sections by immersing them in xylene. The sections were then re-hydrated by incubating them in step wise decreasing concentrations of ethanol, to allow subsequent staining with H&E which are water soluble.

These steps are summarised below:

1. Incubate slides at 60^{o}C 35 min
2. Xylene 3 min X 2
3. 100% Ethanol 3 min X 2
4. 95% Ethanol 3 min X 2
5. 90% Ethanol 3 min
6. 70% Ethanol 3 min
7. dH_2O 3 min

The sections were then stained with Harris Haematoxylin (Haematein), which binds to nucleic acids, and any excess stain removed by washing the sections gently by placing them in a sink with running water, detailed below:

8. Harris Haematoxylin 2 min
9. Running water ~3 min

After staining with Haematoxylin the sections were then counter stained with Eosin which binds to basic cellular and extracellular matrix proteins:

10. 1% Eosin 2 min
11. Running water ~3 min

The sections were then dehydrated by incubating the sections in step wise increasing concentrations of ethanol.

12.	70% Ethanol	30 s
13.	90% Ethanol	30 s
14.	100% Ethanol	30 s X 2
15.	Xylene*	1 min X 2

* Clean xylene was used to prevent wax from the de-waxing process from sticking to the sections and thereby obscuring fields of view.

One drop of DPX mount (VWR) was placed over the tissue section using a Pasteur pipette and a cover slip (VWR) gently lowered over tissue section. The DPX was allowed to dry before visualising slides.

7.3.2 Anti-collagen antibody analysis

Serum IgG_{2a} and IgG_1 anti-collagen antibodies titres were measured by Enzyme-Linked Immunosorbent Assay (ELISA) following the general ELISA protocol as described in 7.5.1. Plates were coated with the indicated concentration of the type II collagen used for immunisation (MD Biosciences). Serum samples were serially diluted 1:2 with assay diluent and optical density measured at 450 nm.

7.3.3 Lymph node intracellular cytokine analysis

For intracellular cytokine staining inguinal and popliteal lymph nodes were removed and single-cell suspensions obtained by crushing the cells through a sterile monofilament filter cloth (100 μM- Cadisch). The number of viable cells (assessed by Trypan Blue staining) were counted and resuspended at 1×10^6/ml in complete media and stimulated overnight with phorbol myristate acetate (PMA - 50 ng/ml, Sigma) and Ionomycin (500 ng/ml, Sigma) in the presence of Golgi-Plug (1 mg/ml, BD Biosciences) which was added 1 hr after stimulation with PMA/ Ionomycin.

After incubation the cells were washed by addition of PBS and centrifugation at 1200 RPM (300 g) and each sample split into three tubes containing approximately 3 X 10^5 cells/ tube. On the first sample an additional three tubes, were used for purposes of compensation (Table 7.2). The cells were then stained with 2 µl/ tube CD4 FITC or isotype control in 50 µl mouse Fc block (BD Biosciences), to inhibit non-specific interactions through the Fc portion of antibodies used for staining, and incubated at 4oC for 30 min in the dark. The cells were then washed with 2% FCS/ PBS and the supernatant discarded followed by incubation with 100 µl/ tube of cytofix/ cytoperm (kit supplied by BD Biosciences), to fix and permeabilize the cells, on ice for 20 min. The cells were washed in perm wash and then stained with anti-IL-17 PE, anti-IFNγ PE or isotype control (all BD Biosciences) and incubated at 4oC for 20 min. The cells were then washed and resuspended in 200 µl 2% FCS/ PBS. The cells were analysed on a FACS Calibre (BD Biosciences) using Cell Quest software TM.

Intracellular cytokine staining
1. Unstained
2. IgG FITC, IgG PE
3. CD4 FITC, IgG PE
4. IgG FITC, IFNγ PE
5. CD4 FITC, INFγ PE
6. CD4 FITC, IL-17 PE

Table 7.2 Stainig for intracellular cytokines
The different combination of stains used for staining of intracellular cytokines on $CD4^+$ T cells. Anti IL-17 PE and anti IFNγ PE share the same isotype.

7.3.4 PCR Genotyping

7.3.4.1 Preparation of tail tips for genotyping

Mice were anaesthetised with isofluorane. Once deeply anaesthetised a 5 mm tail-tip was cut and the tail cauterised.

The tail-tip was placed in 100 µl tail-tip lysis buffer and incubated overnight at 55^{o}C. After the overnight incubation 500 µl of DNase free water (Ambion) was added to the tail-tip solution and incubated at 100^{o}C for 10 minutes. The tubes were then placed on ice and once cooled the tail-tip solution was then centrifuged at 13000 RPM for 5 min. The supernatant was then placed in a fresh tube to be used for genotyping by polymerase chain reaction (PCR).

7.3.4.2 PCR genotyping

The following conditions and primer sequences (designed by Schering- Plough) were used for genotyping of the respective LXR null mice. The primers (synthesised by VH Bio) were reconstituted in Diethyl Pyrocarbonate (DEPC) treated water at a stock concentration of 100 µM and then diluted 1:5 with DEPC treated water for a working stock.

LXRα knockout:

The following primers were used for PCR genotyping of LXRα KO mice:

ORG28-2 5'-TAGACACGGATGATTTGG

ORG028-18 5'-GGAACTCACTATGTAGACC

1µl of each primer working stock (0.4 µM final) was added to a 45 µl PCR master mix (Thermo Scientific) 2.5 mM MgCl2 (final concentration) and made up to 50 µl with DEPC water (Ambion) and the PCR product amplified using on a thermal cycler (Eppendorf) using the setting below:

1. 95^{o}C, 10 min
2. 95^{o}C, 30 s
3. 59^{o}C, 30 s
4. 72^{o}C, 1 min – repeat steps 2 to 4 35 times
5. 72^{o}C, 10 min
6. 4^{o}C, 24 hr

The primers should amplify an 880 bp from the wild-type allele or a 470 bp PCR product from the Cre-excised allele.

LXRβ knockout:

The following primers were used for PCR genotyping of LXRβ KO mice:

ORG029-98 5'-GTCACGAAGCAGCCTGCTGAAC

ORG029-99 5'-GTTAGGATCTCCCATGATAAGAG

ORG029-101 5'-TGAGGCCCAGGCTAGAGGTT

1µl of each primer working stock (0.4 µM final) was added to a 45 µl PCR master mix (Thermo Scientific) 1.5 mM $MgCl_2$ (final concentration) and made up to 50 µl with DEPC water (Ambion) and the PCR product amplified using on a thermal cycler (Eppendorf) using the setting below:

1. 95°C, 10 min
2. 95°C, 30 s
3. 55°C, 30 s
4. 72°C, 1 min – repeat steps 2 to 4 35 times
5. 72°C, 10 min
6. 4°C, 24 hr

The primers should amplify a 437 bp product from the wild-type allele or a 640 bp PCR product from the Cre-excised allele.

7.3.4.3 Agarose gel electrophoresis

All agarose gels used for PCR genotyping were 2% agarose weight/volume. Agarose gels were made by mixing 3 g agarose (Invitrogen) with approximately 150 ml Tris-acetate-EDTA (TAE) buffer followed by heating in a microwave at full power for approximately three minutes until the agarose was dissolved. To 150

ml agarose/ TAE solution, approximately 5 µl ethidium bromide (Sigma) was added and mixed by gentle shaking. The solution was then poured into an agarose gel cast and allowed to set. The gel was then placed in an electrophoresis tank containing TAE buffer. Approximately 25 µl of the PCR product was then transferred into each well in the agarose gel alongside 5 µg of a 1Kbp DNA ladder (Invitrogen). The PCR products were electrophoresed at 100v for approximately 25 min followed by visualisation under UV light (Gel logic 200 imaging system) and Kodak software.

7.3.5 Preparation of bone marrow macrophages

Mice were culled by a recognised schedule 1 method. The rear legs were removed by cutting through the connective tissue at the top of the tibia and placed on ice. All skin and muscle was then removed to give clean and intact tibia and femurs. In a tissue culture flow hood sterile scissors were used to cut through the top and bottom of each tibia and femur. The bone marrow was then flushed out with complete media using a 19G, 20G or 23G needle and syringe into a Petri dish. Using a 1ml pipette tip the bone marrow was then pipetted up and down several times to generate a single cell suspension and pelleted by centrifugation at 1200 RPM (~350 g) for 5min. The supernatant was then decanted and 500 µl of red blood cell lysis solution (Sigma) added to the cell pellet and incubated at room temperature. After two minutes 5ml of complete media was added and the cells pelleted by centrifugation. The number of viable cells (assessed by staining with Trypan Blue) were then counted using a haemocytometer and then resuspended at a concentration of $7X10^5$ cells/ml in complete media supplemented with 10 ng/ml recombinant human MCSF (Peprotech). Human MCSF is crossreactive in mouse derived cells. $7x\ 10^6$ cells were then incubated in a petri dish for 4 days at 37^oC, 5% CO_2. On the fourth day the media and non-adherent cells were removed and replaced with fresh media supplemented with 10 ng/ml MCSF and incubated for a further 3 days. On day 7 the media was removed and the cells removed by cell scraping using ice cold PBS. The number of viable MCSF matured macrophages were then counted and plated out at the required concentration.

7.4 Human cell and tissue procedures

7.4.1 Patients and clinical samples

Leukocytes were purified from buffy coats or from blood of healthy controls or patients meeting ACR criteria for rheumatoid arthritis, CASPAR criteria for psoriatic arthritis or New York criteria for the diagnosis of ankylosing spondylitis after obtaining written informed consent. All RA patients were receiving DMARD therapy. This clinical component of our study was approved by the Glasgow East Ethics Committee.

7.4.2 Separation of human leukocytes from peripheral blood

If peripheral blood mononuclear cells (PBMCs) were being purified from buffy coats then the blood was first diluted 1:2 with PBS. Blood donated from patients or healthy controls was used neat.

Approximately 10 ml of fresh or diluted blood was layered on top of 4 ml room temperature Histopaque 1077 and then centrifuged 2000 RPM (400 g) for 20 min at room temperature. The mononuclear cell layer was then carefully removed using a pasteur pipette and transferred into a clean 15 ml tube, pooling two cell layers per sample. The cells were then washed by addition of 10 ml cold PEA followed by centrifugation for 5 min at 1200 RPM (200 g) at 4^{o}C. This was continued until all the cells from the same sample were pooled into a single tube. The number of viable cells (assessed by Trypan Blue staining) were counted using a haemocytometer and then diluted to the required concentration. Cells were cultured at 37^{o}C in 5% CO_2 controlled environment and treated as described.

7.4.3 Purification of human CD14+ monocytes and CD3+ T cells

Purification of $CD14^+$ monocytes and $CD3^+$ T cells was carried out following magnetic associated cells sorting (MACS) protocol described by Miltenyi Biotec Inc.

PBMCs were prepared as previously described. The PBMCs were then incubated for 15 min at 4°C in 20 µl of $CD14^+$ or $CD3^+$ human micro beads (Miltenyi) and 180 µl PEA per 1 X 10^7 cells on a MACSmix tube rotator (Miltenyi Biotec). Approximately 10 ml of PEA was then added to wash the cells and centrifuged at 1200 RPM (200g). The supernatant was discarded. The cells were then resuspended in 1-2 ml PEA and the $CD3^+$ or $CD14^+$ cells purified by positive selection on an AutoMACS separator (Miltenyi Biotec) using the possel programme. The positive selection process was then repeated on the purified cells to increase cell purity; typically greater than 97% as assessed by FACS analysis staining for $CD3^+$ or $CD14^+$ cells (see results). The number of viable cells were then counted and diluted to the required concentration. Cells were cultured at 37°C in 5% CO_2 controlled environment and treated as described below.

7.4.4 Assessment of cell purity

FACS analysis was used to that the recommended level of monocyte and T cell purity was being achieved as anticipated when using the Miltenyi MACS method of leukocyte purification. After purification cells were firstly washed by addition of PBS and centrifugation at 1200 RPM (300 g) and each sample split into two tubes per sample containing approximately 1 X 10^6 cells/ tube. Monocytes were stained with CD14-FITC or isotype control and the T cells were stained with CD3-PE or isotype control (all BD Bioscience) and incubated for 15 min at room temperature. The cells were then washed and fixed by addition of 200 µl paraformaldehyde (sigma) and incubated for 10 min at room temperature. The cells were then washed, resuspended in 200 µl PEA and stored at 4°C until analysis. The cells were analysed on a FACS Calibre (BD Biosciences) using cell quest software to determine the percentage of $CD14^+$ or $CD3^+$ cells.

7.4.5 Co-culture of human macrophages and T cells

Co-culturing activated monocytes and T cells was first developed as an *in vitro* model of inflammation by Vey *et al* in 1992 in which they demonstrated the secretion of IL-1ß was dependent upon interactions between THP-1 cells and phorbol myristate acetate/ phytohemagglutinin (PMA/PHA) stimulated fixed peripheral blood derived T cells (295). Since its first description this assay is now widely used and is accepted as an *in vitro* model of chronic inflammation which may lead to tissue destruction in diseases such as rheumatoid arthritis (reviewed in (296)).

Human monocytes were purified as described above and resuspended at 5 X 10^5 cells/ml in complete media supplemented with 50 ng/ml (final concentration) recombinant human Macrophage Colony Stimulating Factor (MCSF - PeproTech). The monocytes were cultured in a 96 well plate, 5 X 10^4 cells/ well, in a final volume of 200 µl media for six days.

At the same time syngeneic T cells were purified as described above and resuspended at approximately 2.0 X 10^6 cells/ml in complete media supplemented with recombinant human IL-2 (25 ng/ml), IL-6 (100 ng/ml) and TNF-α (25 ng/ml) (all eBioscience) and cultured at 37^{o}C, 5% CO_2 in T 25 flasks.

After six days the media was removed from the MCSF matured macrophages and discarded. The cytokine activated T (TcKs) cells were washed by addition of PEA and centrifugation at 1200 RPM (200 g) for 5 min. The TcKs were then resuspended at 2.0 X 10^6 cells/ml in complete media and 2.0 X 10^5 cells were transferred into each well containing MCSF matured macrophages; excluding three wells in which macrophages were cultured alone as a control. Similarly, three wells were reserved for culture of TcKs alone. The final volume of media was made up to 200 µl containing LXR agonists at the final concentration as indicated in the results. The TcKs were co-cultured for 24 hrs before supernatants were removed for cytokine analysis and cells were lysed in buffer RLT for TaqMan analysis.

7.4.6 Transwell assays

Human $CD14^+$ monocytes and $CD3^+$ T cells were purified as described above. The macrophages were cultured in a HTS transwell 96 well plate (Corning) and differentiated with MCSF as described above. The $CD3^+$ T cells were stimulated as described above. After six days the media was removed from the macrophages and replaced with 117.5 µl of fresh complete media supplemented with the LXR agonists at the final concentration as indicated in the results. A transwell insert (HTS transwell 96 well permeable support, Corning) was then placed into each well; a transwell with a pore size of 0.4 µm was selected to allow diffusion of molecules but not chemotaxis of cells. The TcKs were then washed, as described above, and resuspended in complete media at a concentration of 5.3 x 10^6 cells/ml. 37.5 µl of cells (2.0 x10^5 TcKs) was then transferred either into the upper or lower compartment so that the TcKS were separated or in co-culture with the macrophages respectively. In all conditions the final volume of media in the lower and upper compartments was made up to 235 µl and 75 µl respectively. LXR agonist or vehicle was added to each compartment at a final concentration as indicated in the results. The cells were then cultured at 37^{o}C, 5% CO_2, for 24 hrs after which the supernatant from each compartment in each well was removed and pooled for cytokine analysis (see below).

7.4.7 LPS stimulation of human CD14+ monocytes

Human monocytes were purified as described above and resuspended at 1 x 10^6 cells/ml in complete media and plated out at 1 x 10^5 cells/well in a 96 well plate. The monocytes were then treated with LXR agonists, see above for details of agonists, at a final concentration between 0.1 µM to 10 µM depending on the agonist as indicated in the results or vehicle (DMSO) and incubated for 24 hrs at 37^{o}C, 5% CO_2. After 24 hrs the media was removed and replaced with fresh complete media supplemented with fresh agonist at the same final concentration and $\pm$ 100 ng/ml LPS (Calbiochem). The monocytes were then cultured for a further 24 hrs after which the culture supernatants were removed for cytokine analysis (see below) and the cells lysed in buffer RLT (Qiagen) for RNA extraction and gene expression analysis (see below).

7.4.8 Dissection of synovial membrane

Synovial membrane was obtained from patients with rheumatoid arthritis or osteoarthritis undergoing synovectomy or joint replacement surgery. Synovial membrane samples were obtained following informed written ethical consent approved by Glasgow East Ethics Committee. Synovial membrane (SM) was dissected out using sterile forceps and scissors and then cut into small pieces of tissue using sterile scalpels. The tissue was then digested with 0.2 mg/ml (final concentration) LPS free collagenase (Liberase LB3, Roche) and incubated at 37^{o}C for 2 hrs in serum free media on a rotator. The digested tissue was then passed through a sterile monofilament filter cloth (100 µm- Cadisch) to make a single cell suspension. The cells were then washed in PEA by centrifugation at 1200 RPM (~200 g) for 5 min and then resuspended in complete media. The number of viable cells were then counted using a haemocytometer and diluted to 2 x 10^6 cells/ml. The cells were cultured at 37^{o}C, 5% CO_2 in a 96 well plate at a density of 2x 10^5 cells/ well and treated as described.

7.5 Cytokine & chemokine analysis

7.5.1 General ELISA protocol

Enzyme Linked Immunosorbent assay (ELISA) was used to measure the concentration of human IL-6, TNFα and IL-23, murine IL-6 and murine anti-collagen IgG1 or IgG_{2a} in cell culture supernatants or serum. The concentrations of antibodies and buffers used for the analysis of each cytokine are shown in Table 7.3.

Immunol microtiter (Thermo Labsystems) plates were coated with detection antibody in the appropriate buffer and incubated overnight at 4^{o}C. The plates were then washed with 0.05% Tween/ PBS followed by addition of blocking buffer, to block non-specific binding, and incubated for 1 hr at 37^{o}C. An eight point standard curve was made using recombinant mouse or human cytokine dissolved in complete media at a top standard concentration of 2,000 pg/ml and serially diluted 1:2. 100 µl of each concentration of standard was added in duplicate to the plate along with two wells containing only media. Samples were

then diluted 1:2-5 with complete media (assay and donor dependent) and 100 µl transferred to each well. The plates were covered and then incubated for 2 hrs at 37^{o}C. The plates were washed with 0.05% Tween/ PBS followed by addition of secondary antibody and incubation at 37^{o}C for 1 hr. The plates were washed and streptavidin HRP diluted 1:1000 with diluent was added to each well and the plates incubated for 1 hr at 37^{o}C. After incubation the plates were washed before addition of 100 µl 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase (Biosource). The reaction was stopped by addition of 100 µl Stop Solution (Biosource) and the mean intensity was read at 450nm on a microplate reader (Dynex Technology).

Cytokine	Coating solution	Blocking solution and diluent	Concentration of primary antibody	Concentration of secondary antibody	Streptavidin HRP dilution	Antibody supplier.
Hs IL-6	PBS	0.5% BSA/ PBS	2 µg/ml(100 µl/ well) (polyclonal)	1.5 µg/ml (100 µl/ well) (polyclonal)	1:1250	Invitrogen
Hs TNFα	PBS	0.5% BSA/ PBS	2 µg/ml (100 µl/ well) (polyclonal)	1.5 µg/ml (100 µl/ well) (polyclonal)	1:1250	Invitrogen
Hs IL-23	PBS	0.5% BSA/ PBS	2 µg/ml (100 µl/ well) (polyclonal)	1.5 µg/ml (100 µl/ well) (polyclonal)	1:1250	Invitrogen
Mm IL-6	0.1 M bicarbonate buffer	10% FCS/ PBS	1 µg/ml: 50 µl/ well (MP5-32C11)	1 µg/ml: 50 µl/ well (polyclonal)	1:1000 (extravidin peroxidase)	BD pharmingen/ Sigma
Mm Anti-collagen IgG1	0.1 M bicarbonate buffer	10% FCS/ PBS	0.4 µg/ml type II collagen (50 µl/ well)	0.5 mg/ml: 50 µl/ well (A19-3)	1:1000 (extravidin peroxidase)	BD pharmingen/ Sigma
Mm Anti-collagen IgG_{2a}	0.1 M bicarbonate buffer	10% FCS/ PBS	0.4 µg/ml type II collagen (50 µl/ well)	0.5 mg/ml: 50 µl/ well (R19-15)	1:1000 (extravidin peroxidase)	BD pharmingen/ Sigma

Table 7.3 Cytokine analysis by ELISA
Human (Hs) and murine (Mm) cytokines were analysed using antibody pairs. The detection antibody was added to each well diluted in the appropriate coating buffer and the secondary antibody added in assay diluent. The final concentration of each antibody and the volume added to each well is shown. All antibodies are monoclonal unless indicated and the clone number is shown in brackets.

7.5.2 Luminex assay

Multiplex cytokine and chemokine analysis of cell culture supernatants or mouse serum concentrations was done by Luminex analysis (Biosource) on a Bio-Plex system (Bio-Rad). Standards were dissolved in 1 ml of assay diluent to reconstitute the each cytokine standard to the required concentration.

Analysis of human cell culture supernatants

For human cell culture supernatants the human cytokine twenty five-plex assay (Biosource) was able to be analyse the following cytokines and chemokines:

Eotaxin, GM-CSF, IFNγ, IL-1RA, IL-1ß, IL-2, IL-4, IL-5, IL-6 IL-7, IL-8, IL-10, IL-12p40/p70, IL-13, IL-15, IL-17, IP-10, MCP-1, MIG, MIP-1α, MIP-1ß, RANTES and TNFα.

Mouse serum analysis

Serum cytokines were analysed using the mouse cytokine twenty-plex (Biosource) which was able to measure the concentration of the following cytokines and chemokines:

FGF, GM-CSF, IFNγ, IL-1α, IL-1ß, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40/p70, IL-13, IL-17, IP-10, KC, MCP-1, MIG, MIP-1α, TNFα and VEGF.

For both mouse and human all samples were diluted 1:2 with assay diluent and 50 µl added to each well, samples were tested in duplicate following the manufacturer's protocol.

7.6 Gene expression analysis

7.6.1 Purification of RNA

DNase digestion was not performed when extracting RNA as all QRT-PCR primers and probes were selected on the basis that they crossed intron/ exon boundaries and would therefore not detect genomic DNA in a TaqMan assay.

7.6.1.1 RNA extraction from tissue

Tissues were dissected from mice, snap frozen and stored at -80^{o}C until required for RNA extraction to maintain RNA stability.

The tissues were placed in a homogeniser tube (Precellys Kit CK28) and 1 ml of Trizol added to each sample. The tissue was then homogenised in a Precellys homogeniser set at 2 X 10 sec at 6500 RPM.

After homogenisation 200 µl of chloroform was added to the sample, vortexed and the contents transferred to a fresh RNase free tube (eppendorf). The tubes were then centrifuged at 13,000 RPM for 20 min at 4^{o}C to separate out the aqueous phase containing the RNA.

The RNA was then precipitated from the aqueous layer by addition of 500 µl propan-2-ol and then pelleted by centrifugation at 13,000 RPM for 20 min at 4^{o}C. The propan-2-ol was then removed and the RNA pellet then washed by addition of 300 µl 70% ethanol and centrifugation at 13,000 RPM for 5 minutes. The ethanol was then removed and the RNA pellet allowed to air dry for approximately 5 min before being dissolved in 300 µl Diethyl Pyrocarbonate (DEPC) treated water.

The RNA was then quantified using a spectrophotometer (Amersham Biosciences) by diluting 1 µl of the RNA in 49 µl (1:50) of water. The stock RNA was then diluted to 0.2 µg/µl with DEPC treated water and stored at -80^{o}C before cDNA synthesis.

7.6.1.2 RNA extraction from cells

RNA extraction from cells cultured in 96 well plates was done following the RNeasy 96 protocol by vacuum technology (Qiagen) as described by the manufacturers. The cells were lysed in 100 µl of buffer RLT, gently agitated to aid cell lysis and stored at -80^{o}C until required for RNA extraction. The RNA was then precipitated by the addition of 100 µl 70% ethanol. The samples were then added to the RNeasy 96 plate to bind the RNA and a vacuum applied to remove

the supernatant. The columns were then washed to remove protein impurities and air dried before eluting the RNA in approximately 60 µl DEPC treated water.

7.6.2 cDNA Synthesis

The following pre-reactions were set up in a thin walled 96 well plate and then covered with a foil lid:

1 µg RNA	5 µl*
Random hexamers 50 ng/µl	1 µl
10 mM dNTP mix	1 µl
DEPC water	3 µl*
Final volume	10 µl

* RNA extracted from tissue or large volume cell cultures was previously quantified and diluted to 0.2 µg/µl using DEPC water. However, if RNA was extracted from cells cultured in 96 well plates then 8 µl of RNA was added and 0 µl DEPC water to maintain a final reaction volume of 10 µl.

The plate was then pulse centrifuged to mix the contents and the RNA denatured in a PCR machine on the following programme:

1. 65°C 5 min
2. 0°C 1 min
3. 4°C Forever

A second master mix was then prepared:

5X RT buffer	4 µl
50 mM MgCl2	2 µl
0.1 M DTT	2 µl
RNase OUT	1 µl

Superscript II RT (200 u/ µl)	0.25 µl
DEPC water	0.75 µl
Final volume	10 µl

The master mix was then added to the contents of each well and mixed by pulse centrifugation. The cDNA was then synthesised by incubating the plates in a PCR machine (eppendorf) on the following programme:

1. 25°C 2 min
2. 42°C 50 min
3. 70°C 15 min
4. 4°C Forever

The cDNA was then diluted 1:3 with DEPC water before using for PCR for analysis of required target genes.

7.6.3 TaqMan QRT-PCR

The cDNA samples were prepared as previously described and assayed in quadruplicate in 10 µl final reaction volumes in a 384 well plate format. All reagents were purchased from Applied Biosystems.

The following controls were required for TaqMan quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR) when synthesising the cDNA:

1. no RNA
2. no template control (only water).

Nb As all primers and TaqMan probes purchased were designed to span from exon to exon over intron gaps no control was required to check for contamination of genomic DNA.

A master mix of the following reagents for each primer and probe set was made; volumes are per QRT-PCR reaction:

QPCR 2X master mix	5 µl
Acid on Demand*	0.5 µl
DEPC treated water	1.5 µl
	7 µl

* Acid on Demand (AoD) = Primer and TaqMan probe mix.

The following AoDs were used for TaqMan analysis of human and mouse cDNA samples (Table 7.4).

Gene name	Species	Catalogue number
Nr1h3 (LXRα)	Human	Hs00172885_m1
Nr1h2 (LXRβ)	Human	Hs00173195_m1
TAF2 (TBP)	Human	Hs00162527_m1
ABCA1	Human	Hs00194045_m1
TLR4	Human	Hs01061963_m1
TBP	Human	4333769F
TBP	Mouse	Mm00446973_m1
Nr1H3 (LXRα)	Mouse	Mm00443454_m1
Nr1H2 (LXRβ)	Mouse	Mm00437262_m1
ABCA1	Mouse	Mm00442646_m1

Table 7.4 TaqMan AoDs: primers and probes.
The details of primer and TaqMan probe mixtures purchased from Applied Biosystems used for TaqMan analysis of samples from humans or mice.

The master mix was then added to a 384 well plate using a 16 channel pipette (7 µl per well). 3 µl of each cDNA sample was then added in quadruplicate and covered with an optical cover. The plate was then centrifuged at 2000 RPM for 2 min to mix the contents. The QRT-PCR reaction was then performed on an Applied Biosystems 17900HT machine for 40 cycles.

The expression of target genes was then quantified relative to the house keeping gene using SDS2.2 software.

7.7 Statistical analysis

All results are displayed ± standard deviation and all statistical analysis was done by students T test or ANOVA test, as indicated in figure legends, using the Graph Pad Prism 4 software. A p value of < 0.05 was considered statistically significant.

8 Activation of the Liver X Receptors potentiates the severity of articular inflammation *in vivo*

8.1 Aims & Introduction

Activation of LXRs has been confirmed by several studies to exert beneficial effects in both the ApoE$^{-/-}$ and the LDLR$^{-/-}$ murine models of atherosclerosis (96, 97, 102). This is mediated primarily by transcriptional upregulation of LXR target genes ABCA1/ G1, ApoE and LDLR which promote reverse cholesterol transport and excretion of cholesterol from the body (49). Many pharmaceutical companies are currently attempting to develop LXR specific agonists for the treatment of metabolic disorders. More recently LXR activation was shown to inhibit the release of the pro-inflammatory cytokine IL-6 from murine LPS stimulated macrophages *in vitro* (117). This study was the first to demonstrate an immuno-modulatory role for LXRs and although a role for LXR activation upon other inflammatory pathways *in vivo* remained unknown these data suggested an anti-inflammatory effect of LXR activation. We therefore hypothesised that whilst LXR agonists may be able to ameliorate atherosclerosis through the modulation of a cholesterol homeostasis they may also exert anti-inflammatory effects and therefore offer potential as a therapy in RA.

The murine CIA model is a widely utilised model of human RA - it is initiated by activation of the immune response to an exogenous antigen that in turn promotes breach of tolerance to self antigen. It allows exploration of components of both the innate and adaptive arms of the immune response (219). The utility of this model has been further exemplified through the development of anti-TNFα therapy which was first suggested to offer therapeutic benefit for RA through studies in murine CIA (199). The CIA model therefore offers the potential to explore the role of LXRs and downstream molecular pathways upon inflammation in arthritis. *I therefore sought to determine the effects of LXR activation upon inflammation generally and the development of arthritis specifically in the murine model of CIA.* However, to ensure activation of LXRs it was first necessary to optimise the route, dose and frequency of agonist administration.

8.2 Optimisation of LXR agonist delivery

8.2.1 Intraperitoneal injection is the optimal route for administration of LXR agonists

Several synthetic LXR agonists, including T1317 and GW3965, have been used in the literature to explore the role of LXRs *in vivo*. However, these compounds were not commercially available and since patents initially prevented the use of GW3965 we were restricted at the outset of my studies to the use of T1317. Furthermore, both compounds had previously been administered by oral gavage as delivery of medications orally is favoured over injection. It was therefore first necessary to determine the optimal route of agonist administration to ensure maximum bioavailability of LXR agonists and subsequent activation of LXRs. Male 7 week old C57BL/6 mice were treated daily with vehicle (5% mulgofen/ PBS) or 10 mg/kg T1317, a dose which had previously been shown to induce LXR activation, by oral gavage, sub-cutaneous injection or intraperitoneal (IP) injection daily for seven days. Mice were culled six hours post final drug administration. The liver and bone marrow were removed to determine the level of LXR transcriptional activation by analysis of ABCA1 expression using Taqman QRT-PCR (Figure 8.1). Administration of T1317 caused an approximate two to three fold increase in the expression of ABCA1 in the liver relative to the vehicle controls by all routes of administration. Similarly, the expression of ABCA1 was upregulated by approximately six and eight fold within the bone marrow by IP or sub-cutaneous injection respectively. However, the expression of ABCA1 in the bone marrow was not altered from basal levels of expression by oral gavage of T1317. This suggests that oral administration does not provide sufficient bioavailability of LXR agonists to all tissues. Since, IP administration of T1317 robustly activated LXRs in both tissues with the least degree of variability IP injection was selected as the method of LXR agonist administration for future *in vivo* studies.

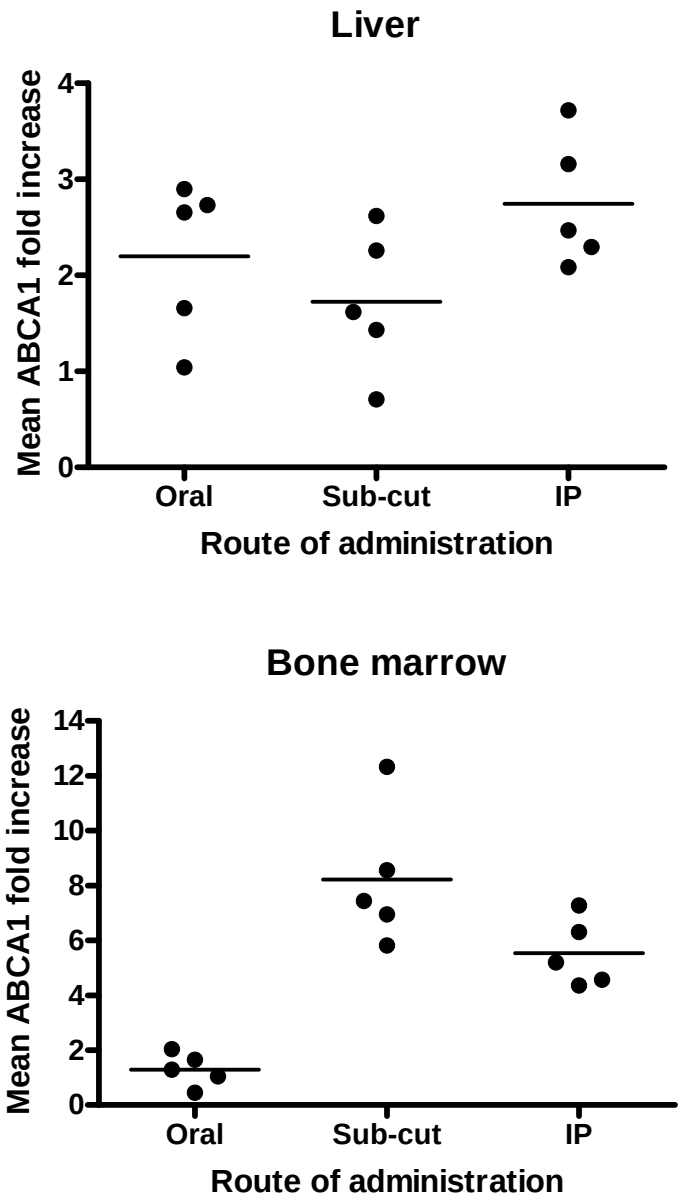


Figure 8.1 Intraperitoneal injection is the optimal route of T1317 administration.
Male mice were treated daily with 10 mg/kg T1317 or vehicle (5% mulgofen/ PBS) by oral gavage, sub-cutaneous injection (sub-cut) or intraperitoneal injection (IP) for 7 days. Mice were culled approximately 6 hrs after final agonist administration and the liver and bone marrow were removed for analysis of ABCA1 expression by Taqman QRT-PCR relative to TBP. Results are displayed as fold increase of ABCA1 expression relative to the respective vehicle group. n = 5/ group.

8.2.2 Optimisation of drug dose

In order to determine the concentrations of T1317 that would induce the activation of LXRs male C57BL/6 mice at 7 weeks of age were treated with vehicle or T1317 at the indicated concentrations by daily IP injection for seven days. Six hours post final drug administration the livers were removed for Taqman QRT-PCR analysis of the expression of ABCA1 expression (Figure 8.2).

The expression of ABCA1 was upregulated by all concentrations of T1317 in a dose dependent manner relative to the vehicle control with the highest level of LXR activation being achieved with a dose of 30 mg/kg T1317. Similarly, the expression of ABCA1 was up-regulated by 3 fold upon treatment with 10 mg/kg T1317 consistent with the previous experiment (Figure 8.1).

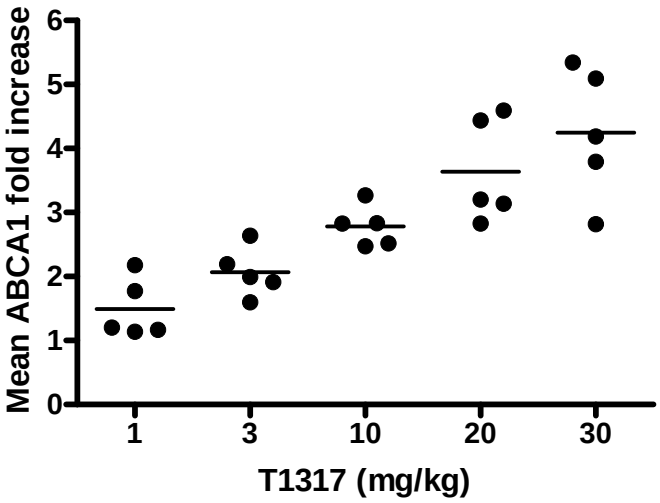


Figure 8.2 T1317 induces the activation of LXRs in a dose responsive manner.
Male mice were treated daily with T1317 or vehicle (5% mulgofen/ PBS) at the indicated concentration by IP injection for 7 days. Mice were culled approximately 6 hrs after final agonist administration and the livers were removed for analysis of ABCA1 expression by Taqman QRT-PCR relative to TBP. Results are displayed as fold increase of ABCA1 expression relative to the vehicle group. n = 5/ group.

LXRs also regulate the expression of FAS which, similar to ABCA1, can also be used to confirm activation of LXR mediated transcription. The expression of FAS was also significantly upregulated in the liver of mice treated with 20 mg/kg and 30 mg/kg T1317 but not at lower agonist concentrations (Figure 8.3). FAS is the rate limiting enzyme in the synthesis of fatty acids and triglycerides and has been shown to be responsible in part for the development of hepatic steatosis in mice treated with LXR agonists (81). This is characterised by the presence of white lipid droplets which can be visualised at the macroscopic level in the liver. In accordance with previous publications the livers of all mice treated with T1317 exhibited signs of hepatic steatosis; this was most prominent in the liver of mice treated with the highest concentrations of T1317 (Figure 8.4).

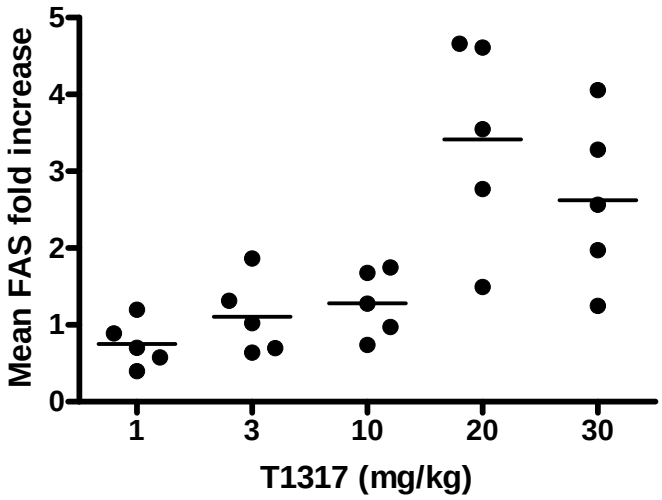


Figure 8.3 LXR activation induces the expression of FAS.
Male mice were treated daily with T1317 or vehicle (5% mulgofen/ PBS) at the indicated concentration by IP for 7 days. Mice were culled approximately 6 hrs after final agonist administration and the livers were removed for analysis of FAS expression by Taqman QRT-PCR relative to TBP. Results are displayed as fold increase of FAS expression relative to the vehicle group. n = 5/ group.

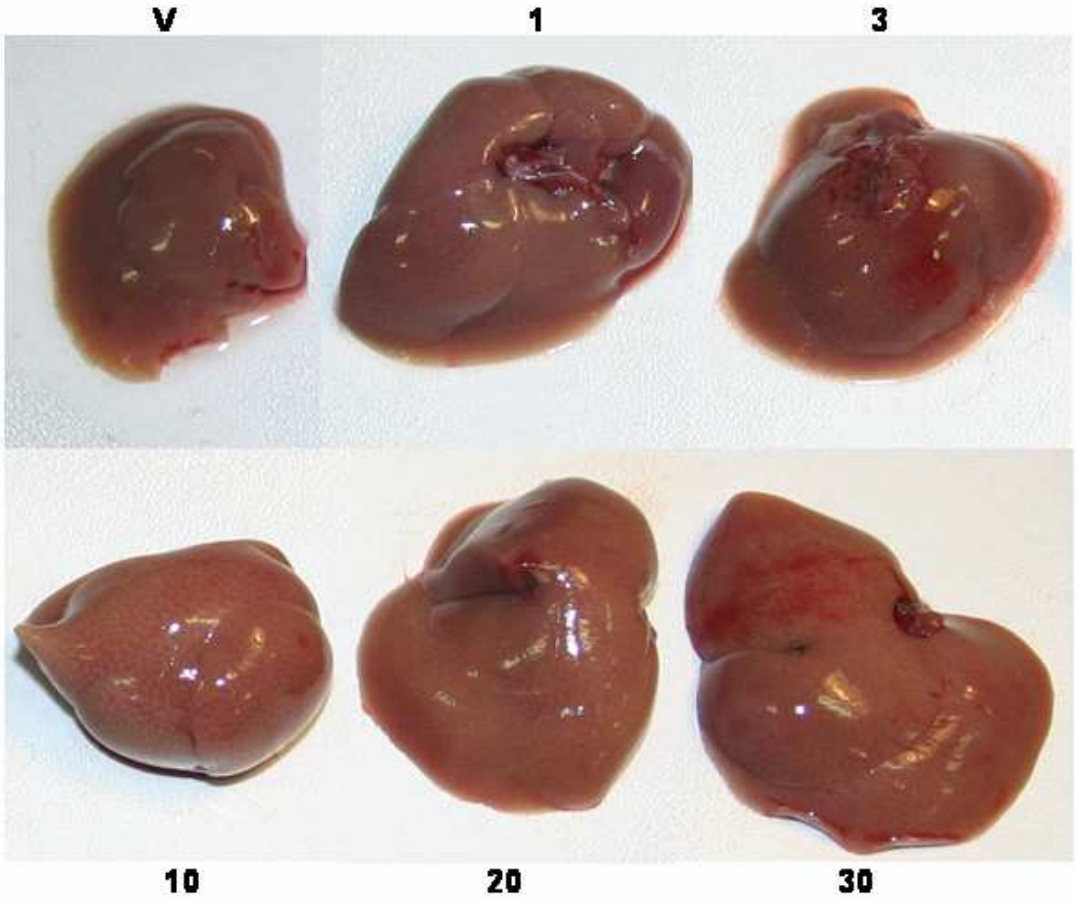


Figure 8.4 Activation of LXRs induces hepatic steatosis.
Livers were removed from mice treated with the indicated concentration of T1317 or vehicle (V- 5%mulgofen/ PBS). The livers of all mice treated with T1317 exhibited hepatic steatosis visualised by the accumulation of white lipid droplets. Representative livers of n = 5/ group.

8.2.3 T1317 is required to be given daily to sustain LXR activation

Early studies shared with us by Schering Plough had indicated that T1317 was undetectable in the circulation of mice within 24 hrs post delivery (data not shown). Therefore, it was important to determine the frequency at which LXR agonist should be administered in order to sustain activation of LXRs *in vivo*. Mice were injected IP with 10 mg/kg T1317 or vehicle and were culled at 6 hrs, 24 hrs and 48 hrs post drug administration. The expression of ABCA1 was determined by Taqman QRT-PCR (Figure 8.5). Similar to previous experiments LXR activation by T1317 induced an approximately 3 fold increase in the expression of ABCA1 6 hrs post agonist delivery. However, at 24 hrs and 48 hrs post administration of drug the expression of ABCA1 had returned to basal levels. This data suggests that daily IP administration of LXR agonist is the minimum frequency required, but also the maximum permitted by the Home Office, to ensure sustained activation of LXRs. Therefore, daily IP injection was selected as the chosen route and frequency of LXR agonist administration for all future experiments.

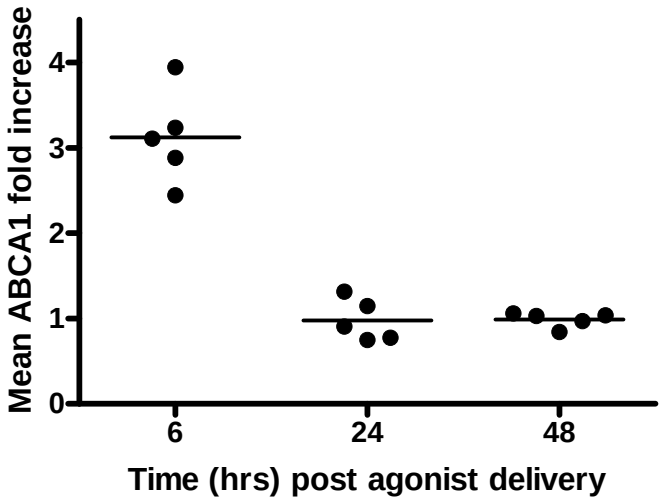


Figure 8.5 T1317 is required to be given daily to sustain activation of LXRs.
Male mice were treated with 10 mg/kg T1317 or vehicle (5% mulgofen/ PBS) by IP. Mice were culled 6 hrs, 24 hrs or 48 hrs after agonist administration and the livers were removed for analysis of ABCA1 expression by Taqman QRT-PCR relative to TBP. Results are displayed as fold increase of ABCA1 expression relative to the vehicle group. n = 5/ group.

8.2.4 Mulgofen does not impact the severity of CIA

T1317 is usually dissolved in the vehicle 5% mulgofen/PBS for *in vivo* delivery. Mulgofen is a detergent which improves drug solubility. To ensure that the mulgofen did not effect the severity of CIA, arthritis was induced in DBA/1 mice treated with PBS or vehicle (5% mulgofen/PBS) by daily IP from day -1 to day 42. Daily administration of vehicle did not significantly alter the clinical score (P = 0.6546) or paw swelling (P = 0.2089) relative to mice treated with PBS (Figure 8.6). Furthermore, there was no significant difference in the concentration of several pro-inflammatory cytokines assayed in serum (Figure 8.7).

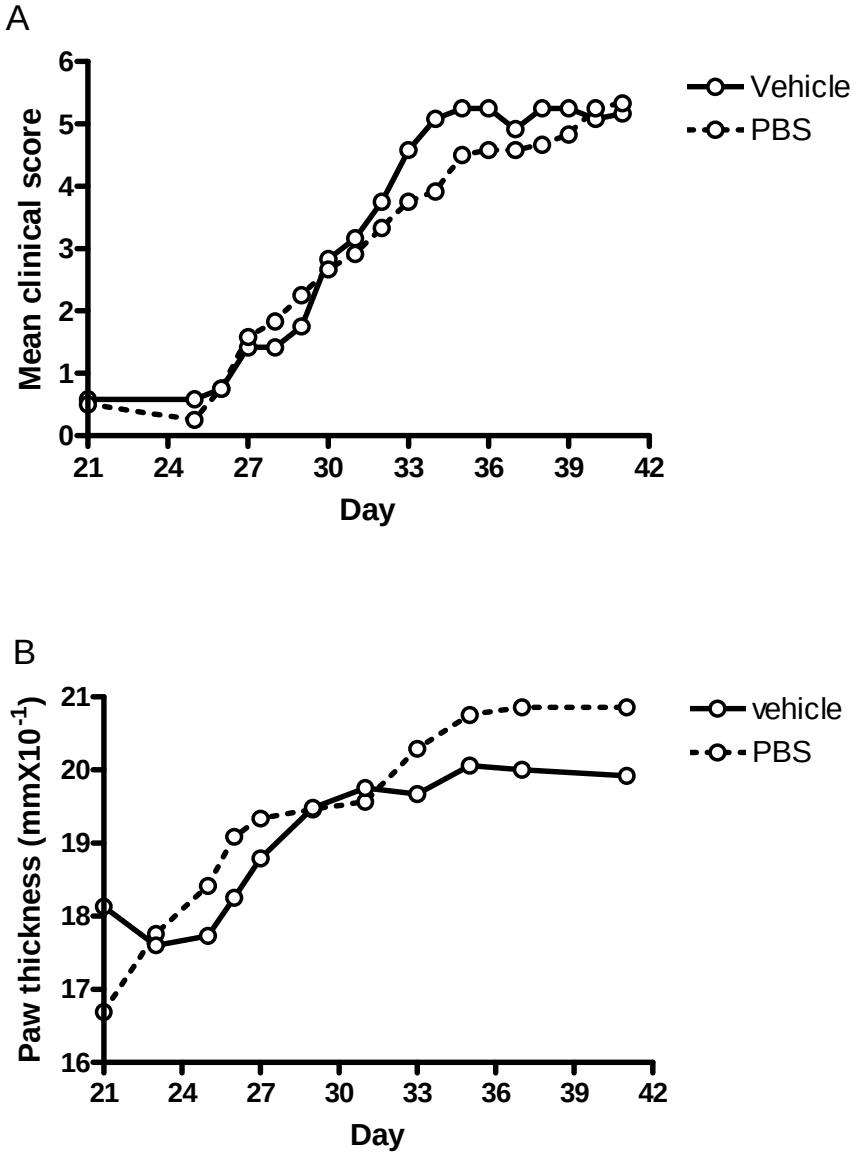


Figure 8.6 Mulgofen does not affect the severity of CIA.
Arthritis was induced in mice which were treated daily IP with vehicle (5% mulgofen/ PBS) or PBS. There was no significant difference in the clinical score (A) or paw thickness (B) between the two treatment groups. Two Way ANOVA; P = 0.2089. n = 12/group.

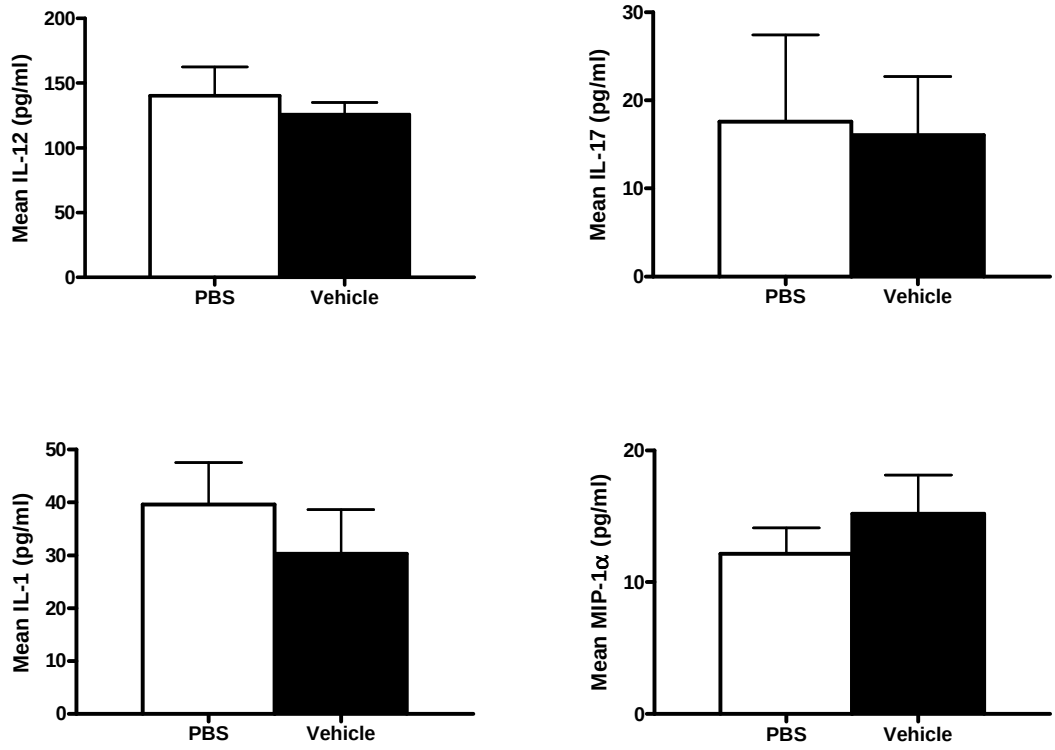


Figure 8.7 Vehicle does not induce the secretion of pro-inflammatory cytokines *in vivo*. Administration of vehicle (5% mulgofen/PBS) to CIA mice does not alter the concentration of serum pro-inflammatory cytokines or chemokines compared to PBS measured by Luminex analysis. n =12/group.

Together these data suggest that activation of LXRs could be achieved in vivo using concentrations of T1317 between 1 mg/kg and 30 mg/kg when administered by daily IP injection; this ensured sufficient bioavailability of agonist and maintained satisfactory activation of LXR transcriptional activity. Furthermore, these data show that the vehicle had no obvious effect upon inflammation and did not affect the severity of murine CIA. Therefore, mulgofen is a suitable vehicle in which to dissolve LXR agonists and explore the role of LXRs in inflammation *in vivo*.

8.3 Identification of a novel pro-inflammatory role for LXRs in vivo

8.3.1 LXR activation by T1317 increases the severity of murine collagen-induced arthritis

8.3.1.1 The clinical severity of arthritis is increased by T1317

Since the delivery of LXR agonists *in vivo* had been optimised it was now possible to investigate whether activation of LXRs by T1317 could modulate inflammation in murine CIA. Arthritis was induced in male DBA/1 mice at approximately 8 weeks of age. Power calculations determined that twelve mice per group was sufficient to detect a 50% change between active and placebo groups with p=0.01. Mice were treated daily IP with 2 mg/kg and 20 mg/kg T1317 or vehicle (5% mulgofen/PBS). In order to investigate the ability of T1317 to affect the development of pre-clinical or established arthritis T1317 was delivered over two time courses; one day prior to the induction of arthritis to day 42 (early treatment regime), or from day 24 post induction of arthritis to 42 (late treatment regime). The experimental design is summarized in (Figure 8.8).

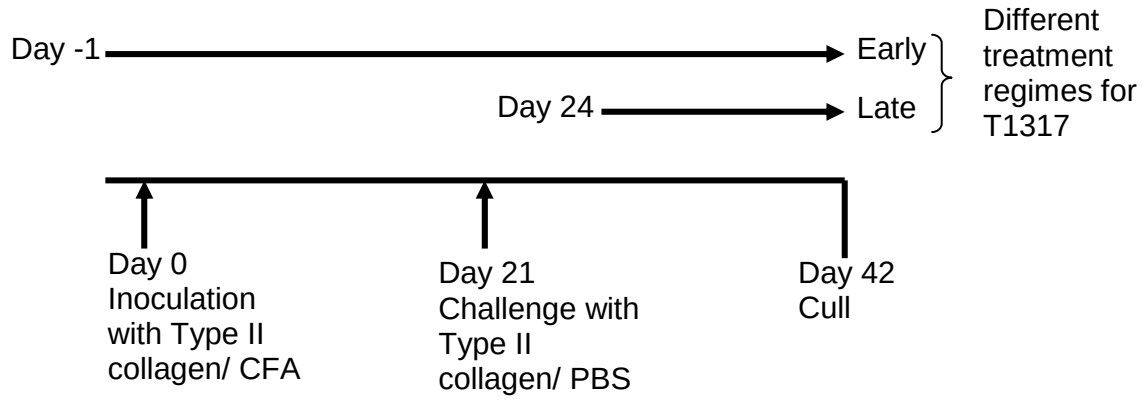


Figure 8.8 CIA experimental design.
Mice were inoculated with 100 µg type II bovine collagen in complete freunds adjuvant (CFA) by an intradermal injection on day 0. At day 21 mice were injected IP with 100 µg type II collagen in PBS. Mice were treated with 2 mg/Kg and 20 mg/Kg T1317 over the two different time courses by daily IP injection. Vehicle (5% mulgofen/ PBS) was administered by daily IP injection over the early time course. n =12/ group.

Administration of T1317 via the early treatment regime induced an earlier onset of disease compared to vehicle recipients (Figure 8.9). From day 21 onwards

mice were monitored daily and assessed for the development of arthritis by the use of a clinical score and measurement of paw swelling. The severity of disease was significantly increased in all the groups receiving T1317 compared to vehicle in a dose dependent manner (Figure 8.10). Furthermore, the groups treated with both 2 mg/kg and 20 mg/kg T1317 on the longer "early" treatment regime exhibited an increased severity of arthritis compared to the respective groups on the shorter "late" treatment regime.

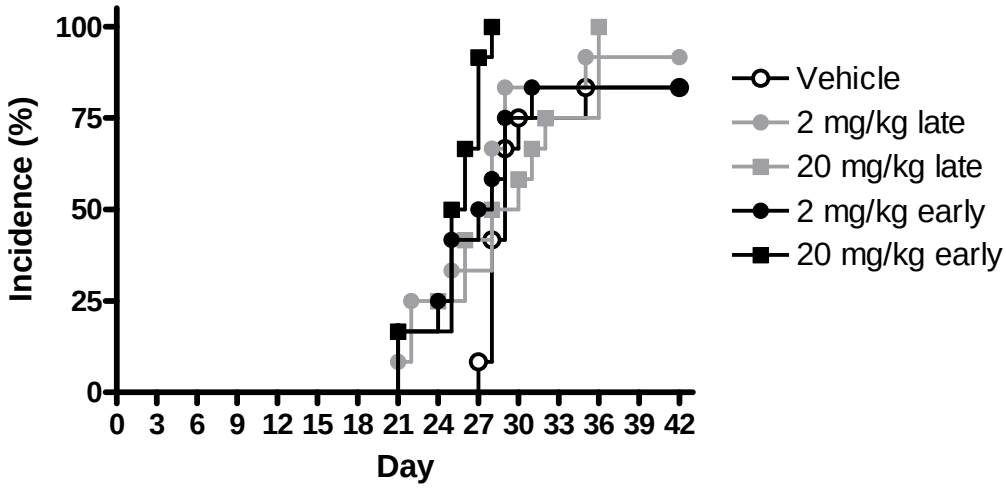


Figure 8.9 Activation of LXRs increases the incidence of arthritis.
Arthritis was induced in male DBA/1 mice and were treated daily with vehicle (5% mulgofen/PBS) or the indicated concentrations of T1317 by IP injection. T1317 was administered over two time course; early: day -1 to day 42 and late: day 24 to day 42. Vehicle was administered by the early treatment regime. n = 12/ group.

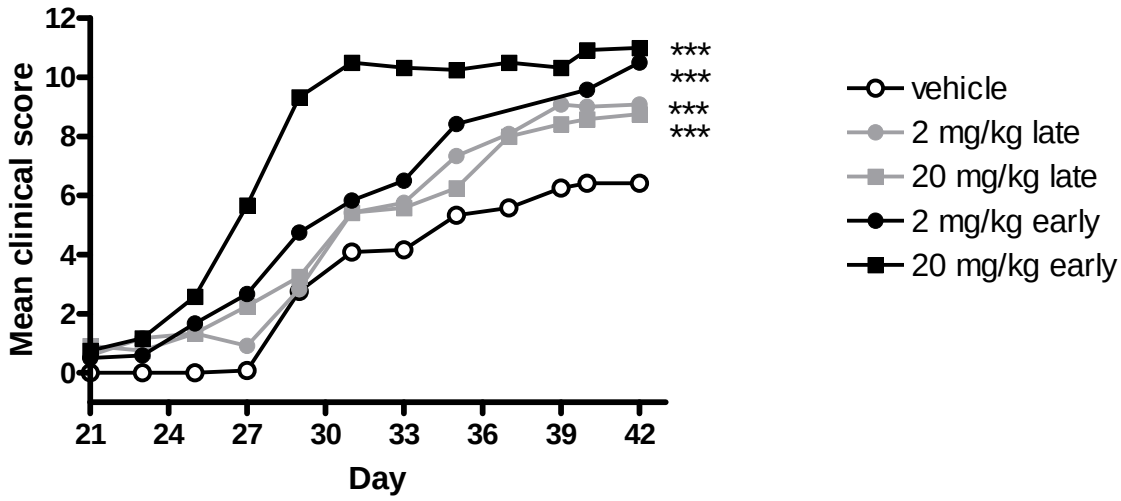


Figure 8.10 The clinical severity of arthritis is increased in mice receiving T1317.
Arthritis was induced in male DBA/1 mice and were treated daily with vehicle (5% mulgofen/PBS) or the indicated concentrations of T1317 by IP injection. T1317 was administered over two time course; early: day -1 to day 42 and late: day 24 to day 42. Vehicle was administered by the early treatment regime. Mice were monitored daily and assigned a clinical score as a measure of arthritis severity. Two way ANOVA, *** $P \leq 0.001$ relative to vehicle; n = 12/ group.

Over the same time period paw swelling was measured as an independent measure of arthritis disease severity (

Figure 8.11). In accordance with the clinical score, paw swelling was significantly increased in all the groups receiving T1317 and did so in a dose dependent manner. Similarly, paw swelling was higher in the groups on the early treatment regime compared with the late treatment regime.

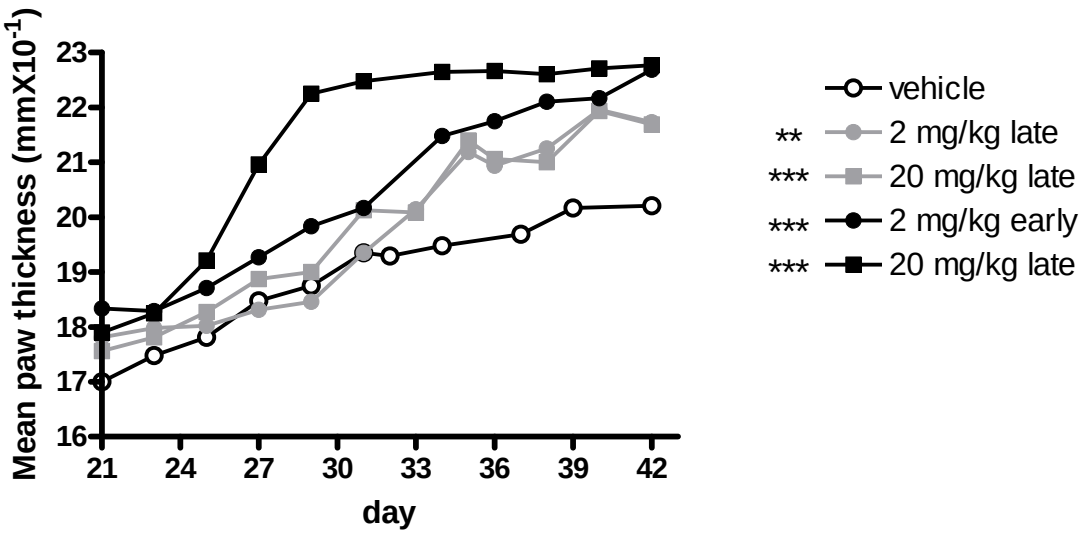


Figure 8.11 Paw swelling is increased in mice receiving T1317.
Arthritis was induced in male DBA/1 mice and were treated daily with vehicle (5% mulgofen/PBS) or the indicated concentrations of T1317 by IP injection. T1317 was administered over two time course; early: day -1 to day 42 and late: day 24 to day 42. Paw swelling was measured as a independent indicator of arthritis severity. Vehicle was administered by the early treatment regime. Two way ANOVA; ** P ≤ 0.01, *** P ≤ 0.001 relative to vehicle; n = 12/ group.

In accordance with the Animals (Scientific Procedures) Act 1986 mice were required to be euthanised if disease severity was too high (maximum score of 12 per animal) or if animals were displaying other signs of general ill health, e.g. weight loss, scruffy coat or sedentary behaviour, as outlined in the appropriate home office project licence. This therefore provided a threshold outcome measure for disease severity with which to compare severe disease in the groups. More mice in the groups receiving T1317, relative to vehicle, were required to be euthanised prior to the planned end of the experiment time course due to the greatly increased level of disease severity (Figure 8.12).

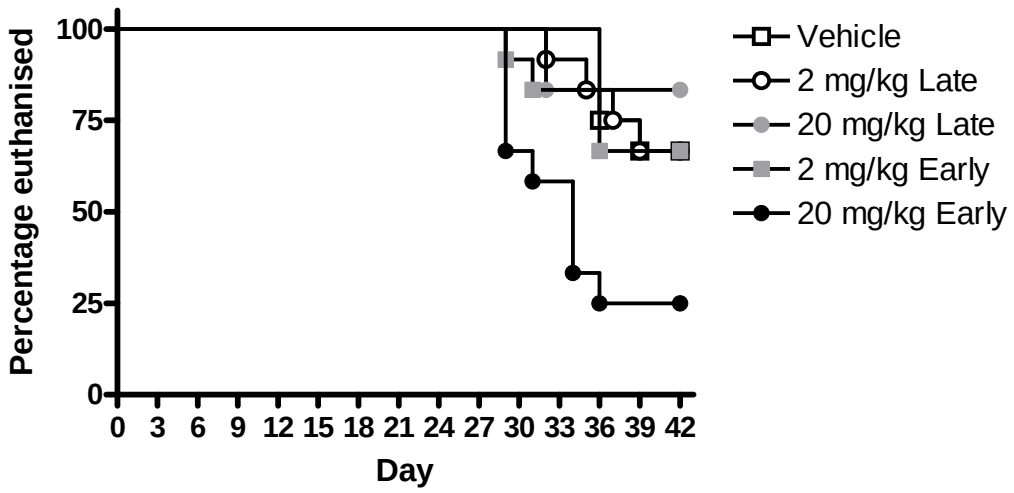


Figure 8.12 The number of mice required to be euthanised is increased with T1317. More mice in the groups receiving T1317 had to be euthanised due to the greatly increased level of disease severity in accordance with the Home Office project licence under the Animals (Scientific Procedures) Act 1986. n = 12/group.

8.3.1.2 T1317 induced the expression of ABCA1 *in vivo*

To confirm that administration of T1317 induced the activation of LXRs the liver was removed from all mice approximately six hours post drug administration and the expression of ABCA1 measured by Taqman QRT-PCR (Figure 8.13). Treatment with 2 mg/Kg or 20 mg/Kg T1317 by both treatment regimes up-regulated the expression of ABCA1 by approximately 2 and 3 fold respectively in comparison to the vehicle control consistent with preliminary experiments (compare to Figure 8.2).

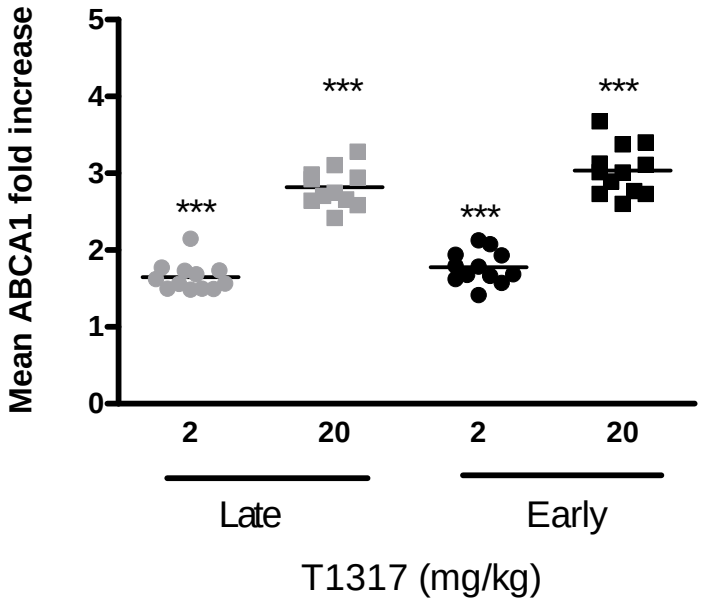


Figure 8.13 T1317 induces LXR activation and the expression of ABCA1 *in vivo.*
To confirm LXR activation in the groups treated with T1317 liver was removed and the expression of the LXR target gene ABCA1 measured by Taqman QRT-PCR normalised to TBP. All groups treated with T1317 exhibited a dose responsive fold increase in the expression of ABCA1 compared to vehicle. n = 12/ group. Students T test; *** P≤ 0.001.

8.3.2 T1317 but not low dose GW3965 increases the severity of murine CIA

Other studies in the literature have previously demonstrated clear anti-inflammatory effects of LXR agonism (117). Therefore, the pro-inflammatory effects of LXR activation in CIA were unexpected and needed to be confirmed. Furthermore, patents that previously prohibited the use of GW3965 had since been withdrawn. GW3965, which is a more specific LXR agonist, could therefore be used alongside T1317 to explore and confirm the effect of LXR agonism upon inflammation in CIA.

8.3.2.1 T1317 but not low dose GW3965 increased the clinical severity of collagen-induced arthritis

To confirm the previous results demonstrating a pro-inflammatory effect of LXR agonism *in vivo* arthritis was induced in male DBA/1 mice at approximately 8 weeks of age, twelve mice per group. Mice were treated daily IP with vehicle (5% mulgofen/PBS), 10 mg/kg and 20 mg/kg T1317 or 10 mg/kg GW3965. T1317 and GW3965 have been shown to induce the expression of ABCA1 at similar Ec50

therefore a concentration of 10 mg/Kg GW3965, similar to T1317 was selected (33, 34). In addition another positive control group was treated with 200 µg/kg dexamethasone to demonstrate that an anti-inflammatory effect could be achieved thereby controlling for operator variability and any adverse effects of multiple IP injections. Since the greatest effect of T1317 was previously seen over the longer treatment period all drugs were administered one day prior to the induction of arthritis to day 41. The experimental design is summarised in (Figure 8.14).

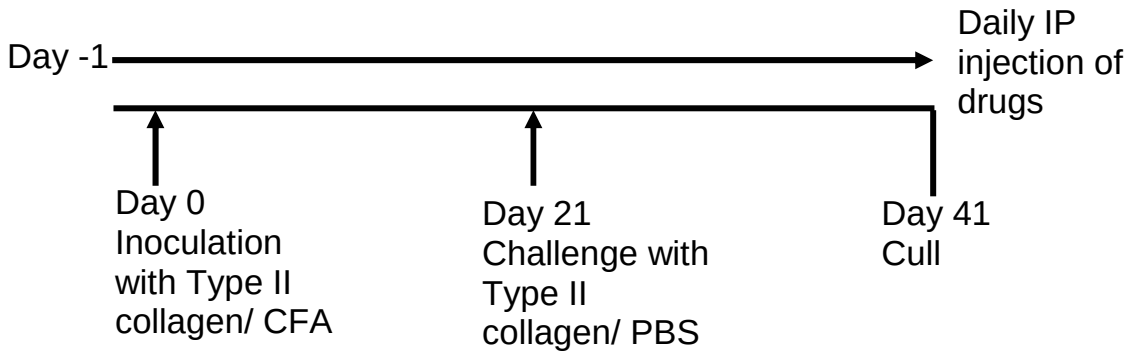


Figure 8.14 CIA experimental design utilising T1317 & GW3965.
Mice were inoculated with 100 µg type II bovine collagen in complete freunds adjuvant (CFA) by an intradermal injection on day 0 and challenged on day 21 IP with 100 µg type II collagen in PBS. Mice were treated daily with vehicle (5% mulgofen/ PBS), 200 µg/Kg dexamethasone, 10 mg/Kg and 20 mg/Kg T1317 or 10 mg/Kg GW3965 from day -1 to day 41 by IP injection. n =12/ group.

Similar to previous results, administration of T1317 and GW3965 resulted in increased incidence of disease compared to vehicle recipients (Figure 8.15). Furthermore, as expected, the group treated with dexamethasone did not develop any clinical signs of disease.

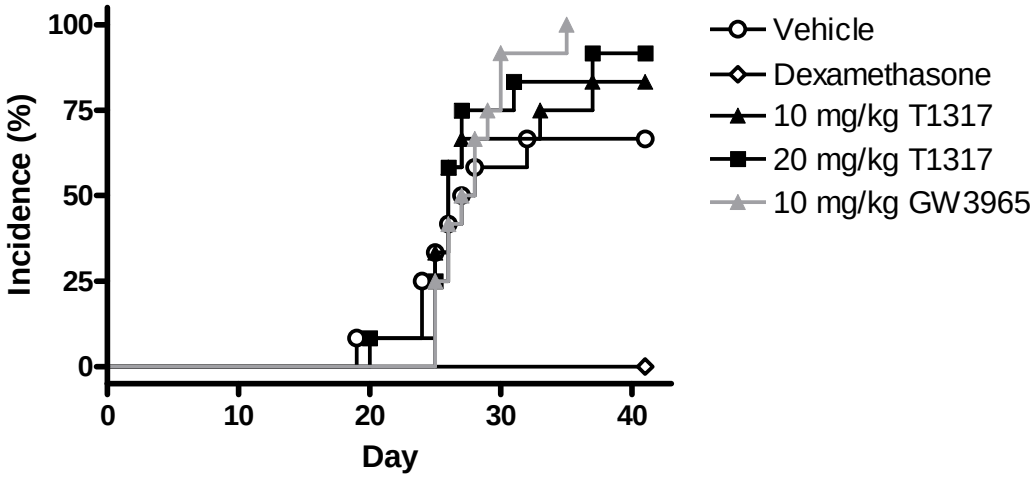


Figure 8.15 Both T1317 and GW3965 increase the incidence of arthritis
Arthritis was induced in male DBA/1 mice which were treated daily with vehicle (5% mulgofen/PBS), 200 µg/kg dexamethasone, T1317 or GW3965 by IP injection from day -1 to day 41. n = 12/ group.

As before, mice were monitored from day 21 onwards for the development of arthritis (Figure 8.16). The development and severity of disease was significantly increased in all the groups receiving T1317 compared to vehicle in a dose responsive manner. The clinical score upon treatment with 20 mg/kg T1317 was comparable with that obtained in prior experiments (compare to Figure 8.10). However, treatment with 10 mg/kg GW3965 did not significantly alter the severity of disease. The clinical scores were also confirmed by two blinded observers at several time points throughout the experiment.

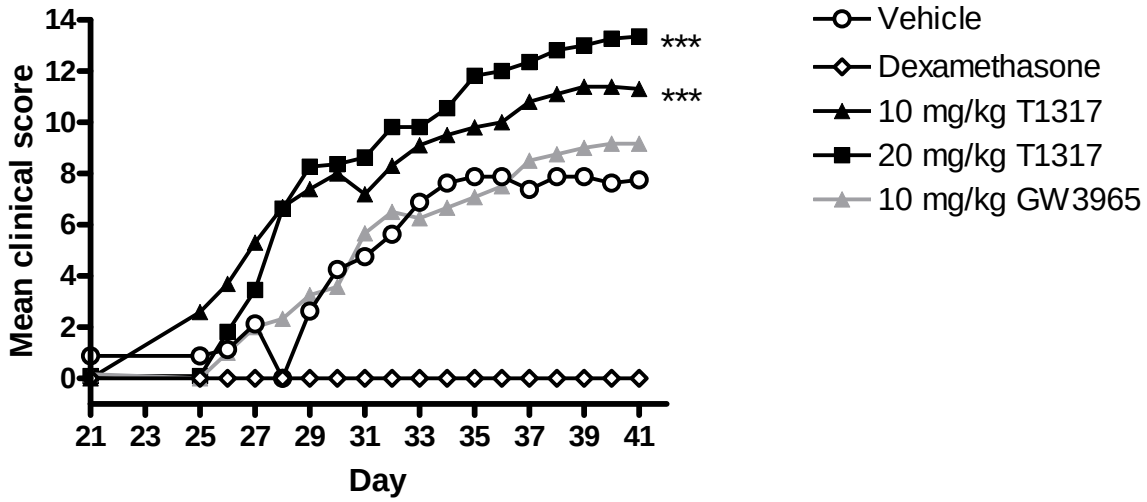


Figure 8.16 T1317 but not low dose GW3965 increases the severity of arthritis
Arthritis was induced in male DBA/1 mice which were treated daily with vehicle (5% mulgofen/PBS), 200 µg/kg dexamethasone, T1317 or GW3965 by IP injection from day -1 to day 41. Mice were monitored daily and assigned a clinical score for the severity of arthritis. Two way ANOVA; *** P ≤ 0.001 relative to vehicle; n = 12/ group.

Similarly, paw swelling was significantly increased in both groups receiving T1317 compared to vehicle (Figure 8.17). Although, paw swelling was greater in the group receiving GW3965 this was not statistically significant (P = 0.06). In accordance with the clinical score there was no increase in the paw thickness in the mice receiving dexamethasone. Moreover, more mice were euthanised in the groups treated with T1317, but not GW3965, compared to vehicle control (Figure 8.18). Together these data indicate that disease severity was only increased in the groups receiving T1317.

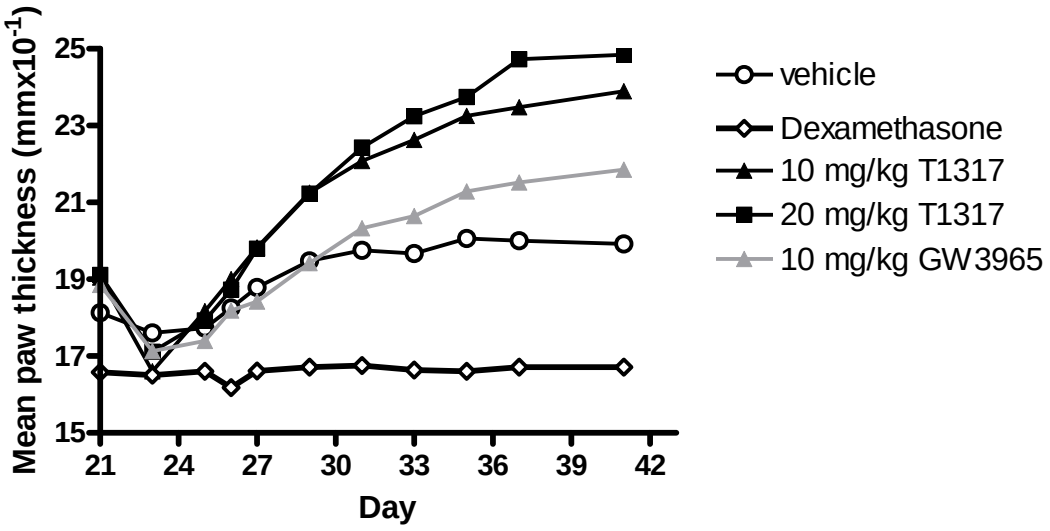


Figure 8.17 Paw swelling is increased by LXR agonism
Arthritis was induced in male DBA/1 mice which were treated daily with vehicle (5% mulgofen/PBS), 200 µg/kg dexamethasone, T1317 or GW3965 by IP injection from day -1 to day 41. Paw swelling was measured as an independent indicator of arthritis severity. Two way ANOVA; ** $P \leq 0.01$; n = 12/ group.

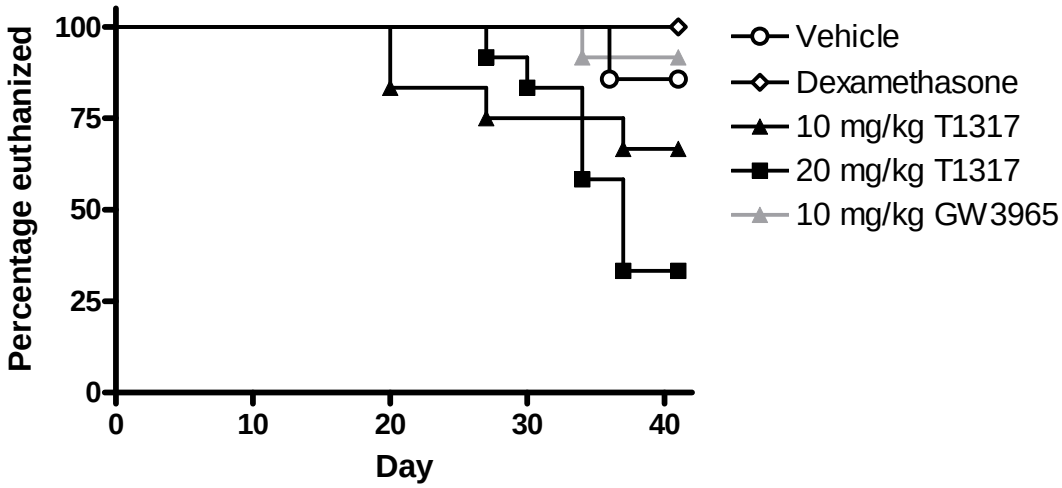


Figure 8.18 Mortality is increased in mice receiving T1317.
Arthritis was induced in male DBA/1 mice which were treated daily with vehicle (5% mulgofen/PBS), 200 µg/kg dexamethasone, T1317 or GW3965 by IP injection from day -1 to day 41. Mice were euthanised if the severity of disease was too severe or displayed general signs of ill health in accordance with the Home Office project licence. Mortality was not altered in mice receiving GW3965 in comparison to vehicle. n = 12/ group.

8.3.2.2 *Ex vivo* assessment of inflammatory cytokines and antibody titres

Upon termination of the experiment blood samples were taken by cardiac puncture for analysis of serum cytokine and chemokine concentrations measured by Luminex analysis (Figure 8.19). The concentration of several serum

proinflammatory cytokines, IL-1, IL-17, IL-12 and GM-CSF, were increased in the groups treated with T1317 in a dose responsive manner. None of these pro-inflammatory cytokines were significantly elevated in response to 10 mg/kg GW3965. However, the anti-inflammatory cytokine IL-10 was elevated in response to treatment with both T1317 and GW3965. Furthermore, treatment with T1317 increased the serum concentration of the chemokine MIP-1α, whilst the concentration of MIG was higher in both the T1317 and GW3965 groups. The concentration of IL-12 was significantly decreased and was the only cytokine affected by dexamethasone. IL-12 supports the polarisation of naïve T cell towards a Th1 phenotype. Lower concentrations of serum IL-12 are indicative of and consistent with findings that steroids ameliorate articular inflammation primarily through the inhibition of T cell proliferation. The cytokines and chemokines IL-2, IL-4, IL-5, IL-6, IL-13, IFNγ, TNFα, FGF, VEGF, IP-10, KC and MCP-1 were not detectable at this time point.

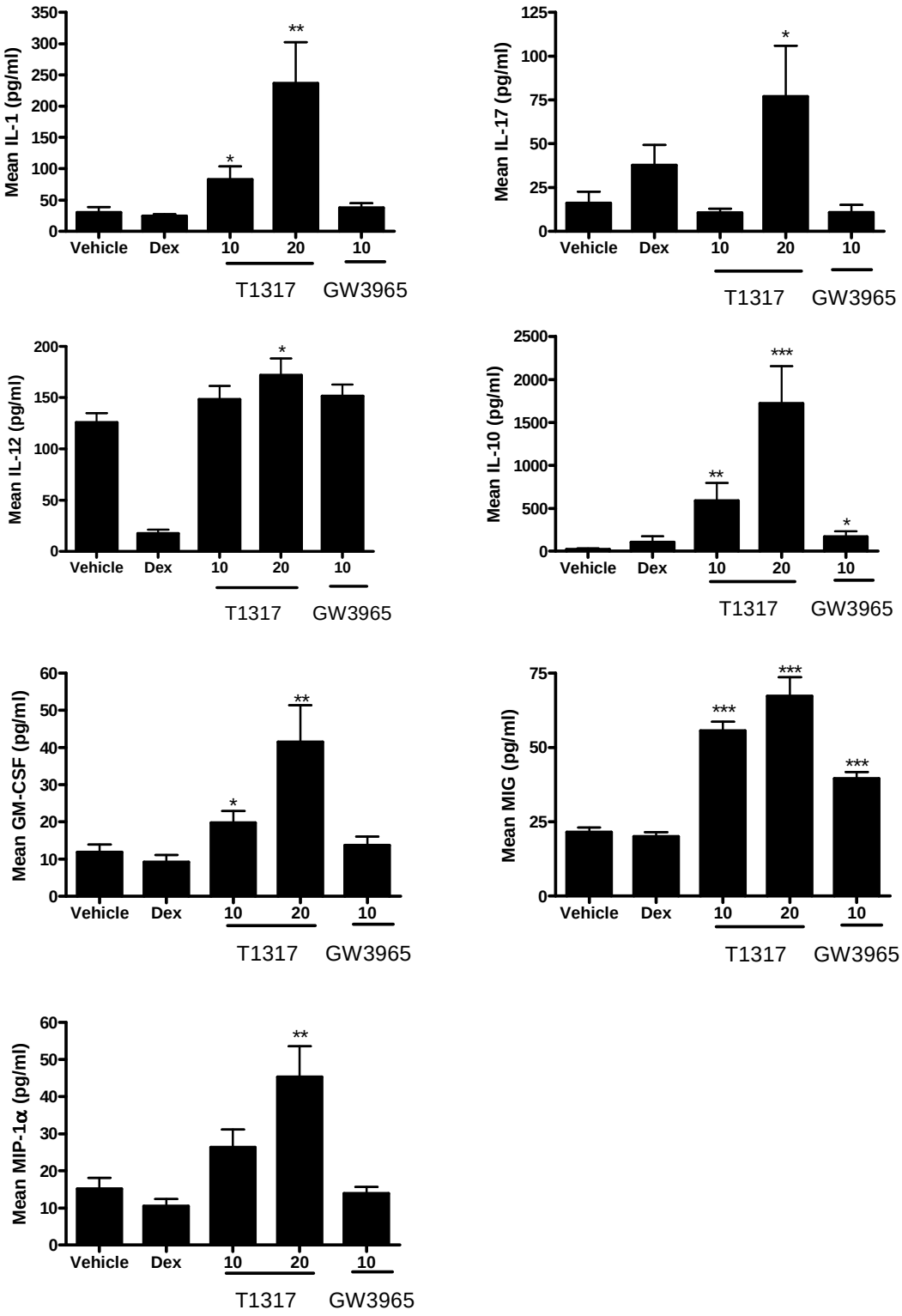


Figure 8.19 T1317 increases the concentrations of serum inflammatory cytokines and chemokines.
Serum cytokine and chemokine concentration from mice at day 41 of the CIA model were measured by Luminex analysis. Mice had been treated daily IP with vehicle (5% mulgofen/ PBS), 200 µg/kg dexamethasone (Dex), T1317 or GW3965. Other cytokines or chemokines were not detectable at this time point. Unpaired Students T test; * P ≤ 0.05, ** P ≤ 0.01 and *** P ≤ 0.001. n =12/group.

To determine the effect of LXR activation by T1317 or GW3965 upon T cell activation the popliteal and inguinal lymph nodes were removed and crushed through a cell strainer to generate a single cell suspension. Very few cells were obtained from the group treated with dexamethasone which precluded analysis of T cell function by this method (Figure 8.20). The cells were stimulated overnight with PMA and Ionomycin and the percentage of $CD4^+$ cells that were $IFN\gamma^+$ (Th1) or $IL\text{-}17^+$ (Th17) was analysed by flow cytometry (Figure 8.21). In the groups treated with T1317, despite a dose dependent trend, no significant increase in the percentage of $IFN\gamma^+$ cells was observed (20 mg/Kg T1317; P = 0.535). However, there was a significant increase in the percentage of $CD4^-$ $IL\text{-}17^+$ cells.

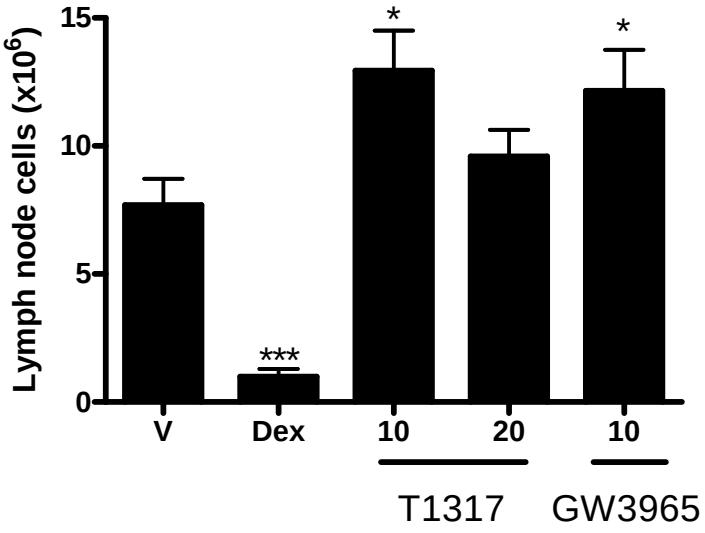


Figure 8.20 The number of lymph node cells is increased by LXR agonism.
The popliteal and inguinal lymph nodes were removed from mice treated with vehicle (V), 200 µg/Kg Dexamethasone (Dex), T1317 or GW3965 (mg/Kg) and crushed through a cell strainer to make a single cell suspension and the total number of viable cells assessed by staining with Trypan blue were counted. The number of cells from the group treated with 200 µg dexamethasone was significantly reduced relative to vehicle control. Unpaired students T test; *** $P \leq 0.001$, * $P \leq 0.05$. n = 12/group.

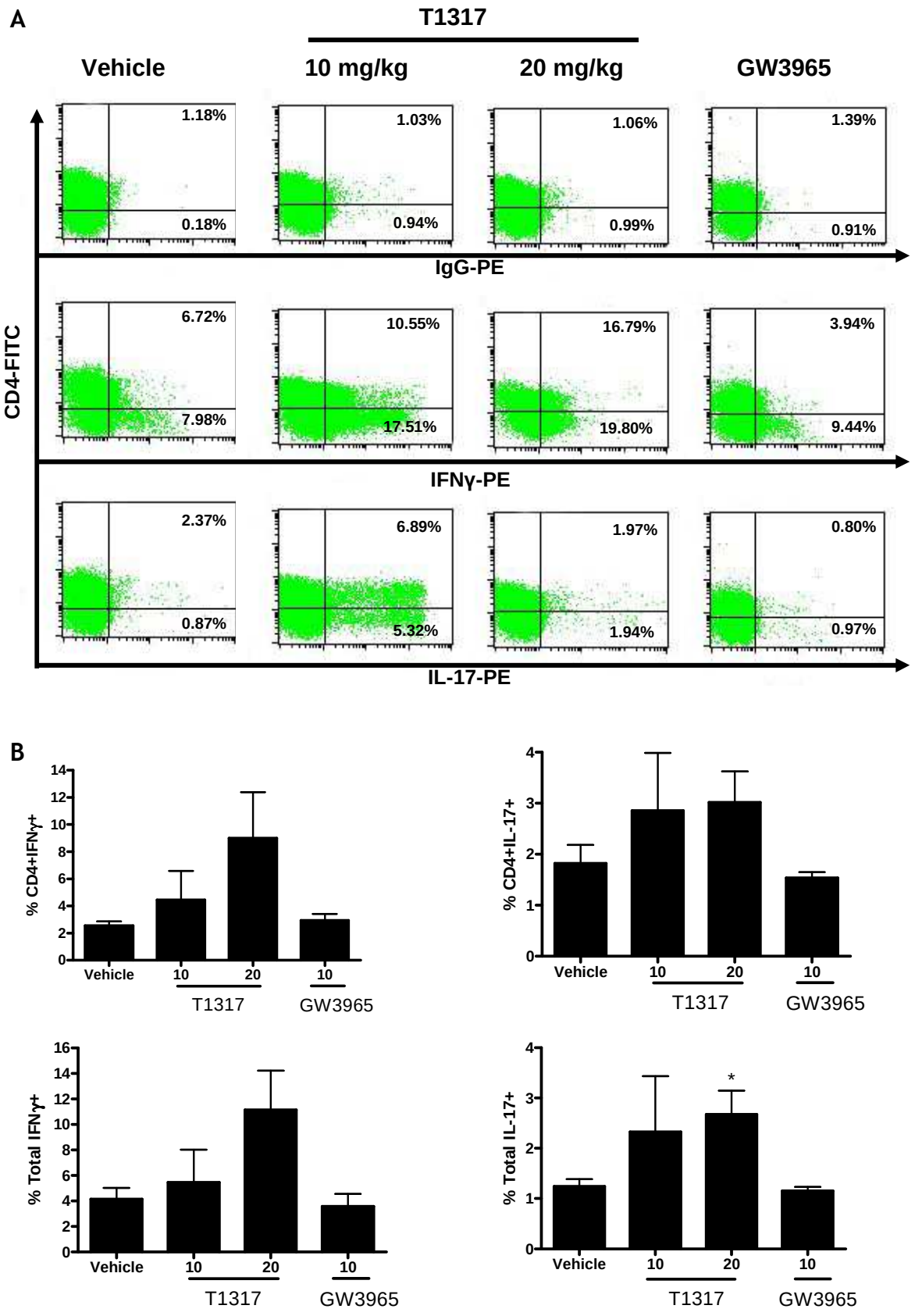


Figure 8.21 Intracellular cytokine analysis of lymph node T cells.
The cells from popliteal and inguinal lymph nodes of mice treated with vehicle, T1317 or GW3965 at the indicated concentration (mg/Kg) were stimulated overnight with PMA and Ionomycin. The percentage of $CD4^+IL\text{-}17^+$ or $CD4^+IFN\gamma^+$ T cells was determined by flow cytometric analysis. (A) Representative images of flow cytometric analysis from each group. (B) Graphical representation of mean percentage IFNγ or IL-17 positive cells from each group. Unpaired Students T test; * $p \leq 0.05$. n = 12/ group.

Murine CIA is associated with anti-collagen antibodies of which IgG_{2a} is the predominant isotype. Since 20 mg/kg T1317 had the largest effect upon the concentration of serum cytokines and chemokines and lymphocytes activation the concentration of serum anti-collagen IgG_{2a} specific antibodies in this group was measured by ELISA (Figure 8.22). However, there was no significant difference in the concentration of anti-collagen antibodies in the group treated with 20 mg/Kg T1317 relative to vehicle at this time point.

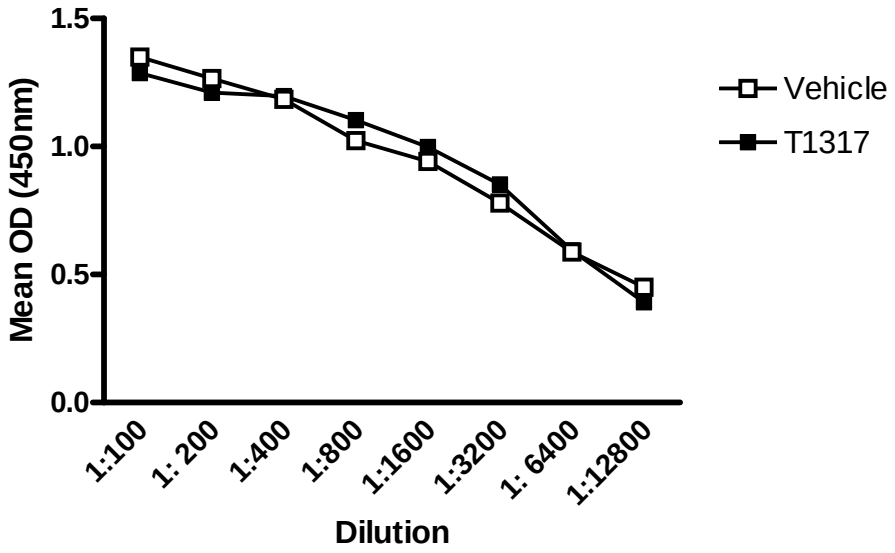


Figure 8.22 T1317 does not alter serum anti-collagen IgG_{2a} antibody titres. Serum from mice treated with vehicle of 20 mg/Kg T1317 was serially diluted and the concentration of anti-collagen IgG_{2a} antibodies was measured by ELISA. n = 12/group.

8.3.2.3 T1317 and GW3965 induce activation of LXRs

Since treatment of mice with 10 mg/Kg T1317 or 10 mg/Kg GW3965 had different effects upon the development of arthritis disease severity it was essential to ensure that both compounds had induced the transcriptional activation of LXRs (Figure 8.23). In previous experiments the expression of ABCA1 had previously been measured in the liver. However, data from Schering Plough indicated that GW3965 does not readily induce the expression of ABCA1 in the liver whereas both T1317 and GW3965 are able to up-regulate ABCA1 expression in the jejunum (data not shown). The expression of ABCA1 at six hours post drug administration was upregulated approximately 10 fold in the groups treated with T1317 and GW3965 as measured by Taqman QRT-PCR

relative to vehicle control. Treatment with dexamethasone did not alter LXR mediated expression of ABCA1.

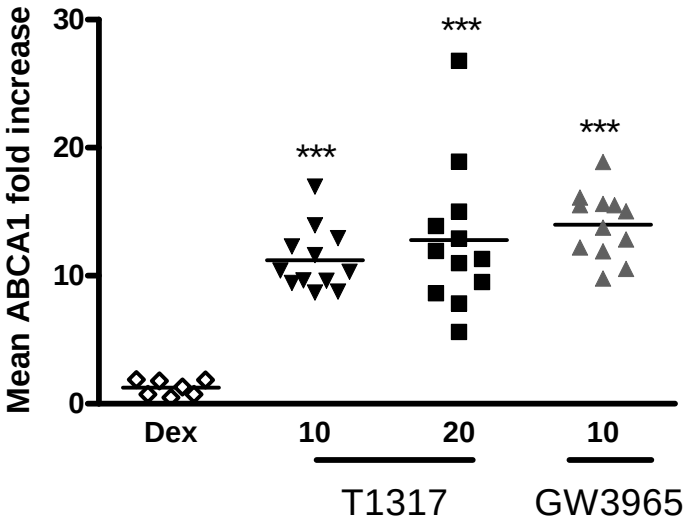


Figure 8.23 Induction of LXR activation by T1317 and GW3965.
The expression of the LXR reporter gene ABCA1 was measured by Taqman QRT-PCR in the jejunum of mice treated with 200 µg/kg dexamethasone (dex), T1317 or GW3965 at the indicated concentration (mg/Kg). Results are displayed as mean fold increase of ABCA1 expression relative to vehicle. n = 12/ group. Students T test; *** P ≤ 0.001.

8.3.3 LXR agonism by high dose GW3965 promotes articular inflammation

8.3.3.1 LXR activation by GW3965 increases the incidence and severity of arthritis

The results of the previous two independent experiments have demonstrated that LXR activation by T1317 increases the severity of murine CIA in a dose responsive manner but not by a lower concentration of GW3965. To address the effects of GW3965 administration at a higher concentration, arthritis was induced in male DBA/1 mice at approximately 8 weeks of age. Mice were treated daily IP with vehicle (5% mulgofen/PBS) or 30 mg/kg GW3965. Drugs were administered from one day prior to the induction of arthritis to the expected peak of disease at day 31. The experimental design is summarised in (Figure 8.24). Treatment with 30 mg/Kg GW3965 dramatically increased the incidence of disease compared to vehicle recipients (Figure 8.25).

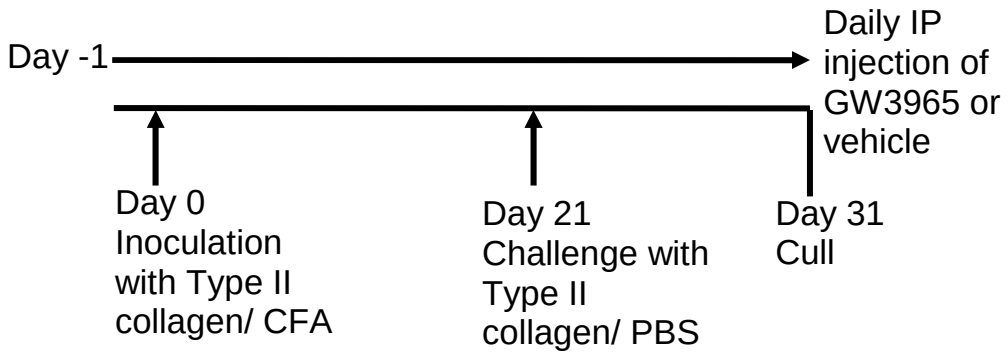


Figure 8.24 CIA experimental design utilising 30 mg/Kg GW3965.
Mice were inoculated with 100 µg type II bovine collagen in complete freunds adjuvant (CFA) by an intradermal injection on day 0 and challenged on day 21 IP with 100 µg type II collagen in PBS. Mice were treated daily with vehicle (5% mulgofen/ PBS or 30 mg/Kg GW3965 from day -1 to day 31 by IP injection. n =12/ group.

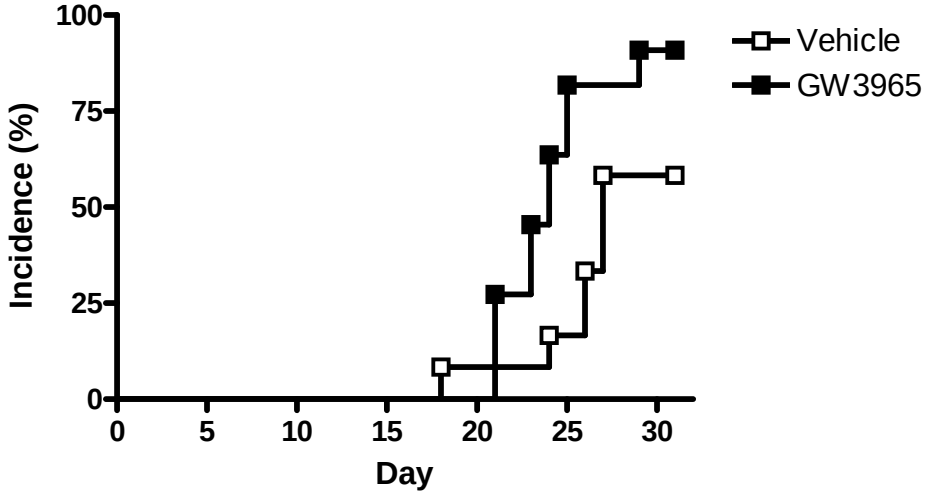


Figure 8.25 GW3965 increases the incidence of arthritis.
Arthritis was induced in male DBA/1 mice and treated daily by IP injection with vehicle (5% mulgofen/ PBS) or 30 mg/kg GW3965 from day -1 to day 31. n =12/group.

Similar to previous experiments, mice were monitored for clinical signs of disease. Treatment with 30 mg/Kg GW3965 significantly increased the clinical severity of arthritis (Figure 8.26). In agreement with the clinical score the extent of paw swelling was also greater in the group receiving 30 mg/Kg GW3965 (Figure 8.27). Due to the short period of time in which disease was allowed to develop and the lack of time points the difference in paw swelling was not significant by Two Way ANOVA. However, the individual time points were significantly different by Students unpaired T test (not shown).

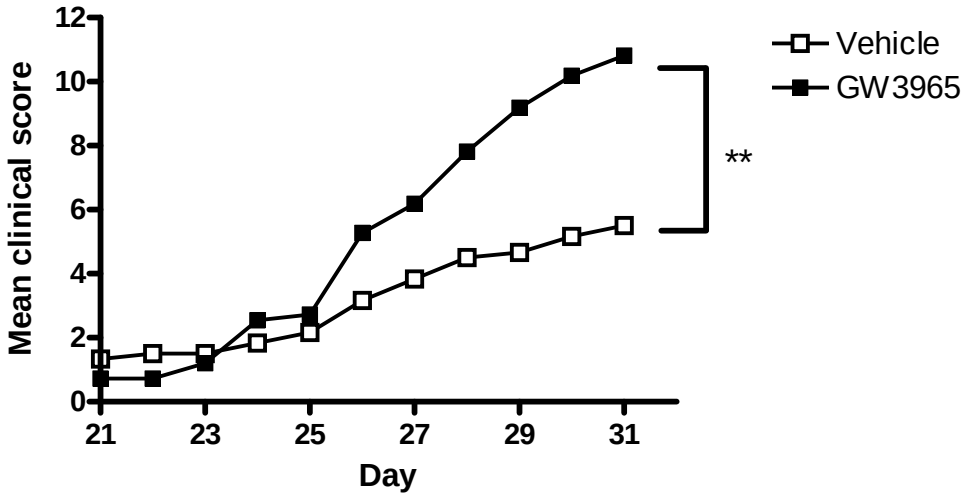


Figure 8.26 GW3965 increases the clinical severity of arthritis.
Arthritis was induced in male DBA/1 mice and treated daily by IP injection with Vehicle (5% mulgofen/ PBS) or 30 mg/kg GW3965 from day -1 to day 31. Mice were monitored and assigned a clinical score as a measure of disease severity. Two Way ANOVA; ** P ≤ 0.01. n =12/ group.

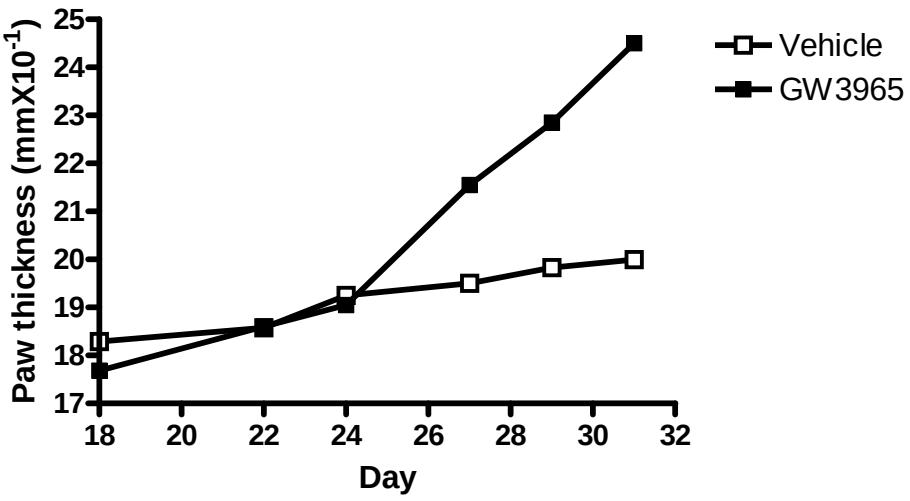


Figure 8.27 Paw swelling is increased by treatment with GW3965.
Arthritis was induced in male DBA/1 mice and treated daily by IP injection with Vehicle (5% mulgofen/ PBS) or 30 mg/kg GW3965 from day -1 to day 31. Mice were monitored and the extent of paw swelling measured as an independent indicator of disease severity. The increase in paw swelling was not significant by Two Way ANOVA but the individual time points were significantly different by unpaired T test (not shown). n =12/ group.

Upon termination of the experiment paws were fixed, decalcified and stained for histological analysis of articular inflammation and erosion (Figure 8.28). In the group receiving 30 mg/Kg GW3965 histological analysis revealed a marked inflammatory infiltrate, cartilage erosion and hyperplasia of the synovial

membrane. The overall extent of inflammation and erosion was significantly increased in the groups treated with GW3965.

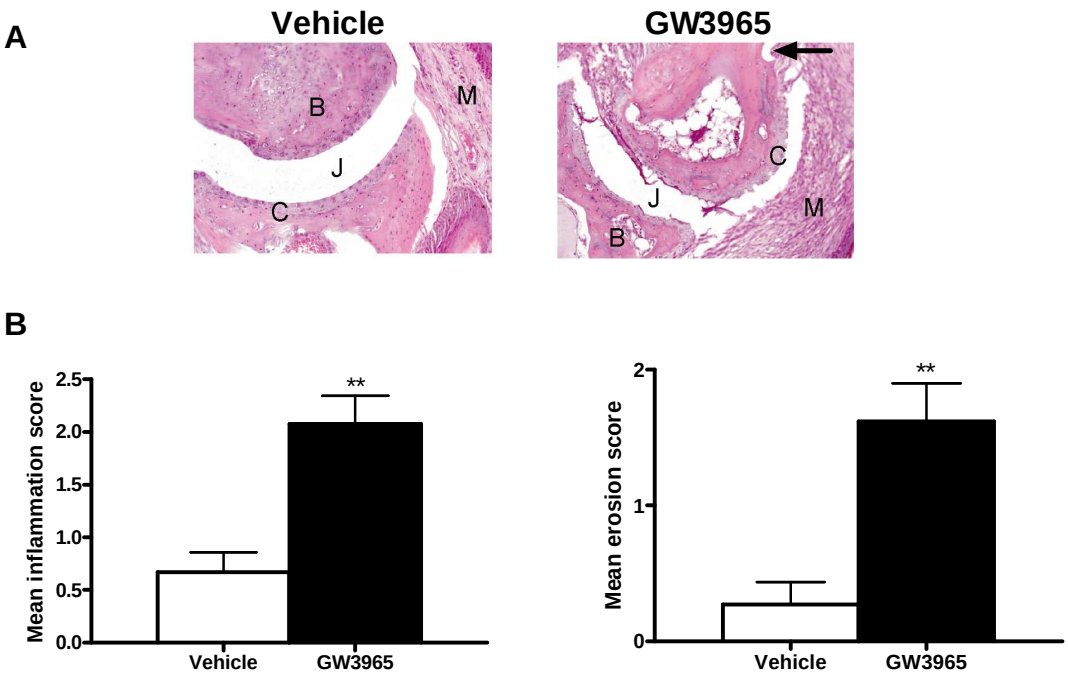


Figure 8.28 Histological severity of arthritis is increased by GW3965.
(A) Representative H & E staining of joints from mice receiving vehicle (5% mulgofen/ PBS) or 30 mg/kg GW3965. Photos at 10X magnification. Abbreviations; B = bone, C = cartilage, J = joint space and M = synovial membrane. Bone erosion is indicated by the arrow (B) Administration of 30mg/kg GW3965 (black bar) increases the amount of inflammatory infiltrate and the severity of erosion relative to vehicle (open bar). Mann Whitney-test; ** P < 0.01. n = 12/ group.

8.3.3.2 *Ex vivo* cytokine and anti-collagen antibody analysis

Previously it was necessary to euthanize mice prior to completion of the full experimental time course which introduced a degree of variability in the results of *ex vivo* cytokine analysis. Therefore the shorter experimental time course was chosen to allow analysis of cytokines at the peak of disease and remove variability caused by individuals being culled at multiple time points. Multiple pro-inflammatory cytokines, IL-1, IL-6, IL-13, IL-17, GM-CSF, and inflammatory chemokines, MIP-1α, MCP-1, MIG, IP-10 and KC, were higher in the group treated with 30 mg/Kg GW3965 compared to vehicle controls (Figure 8.29 and Figure 8.30). In addition, there was no significant difference in the concentration of serum IL-10, IL-12 and FGF between the two groups. IL-2, IL-4, IL-5, TNFα and VEGF were not detected at this time point.

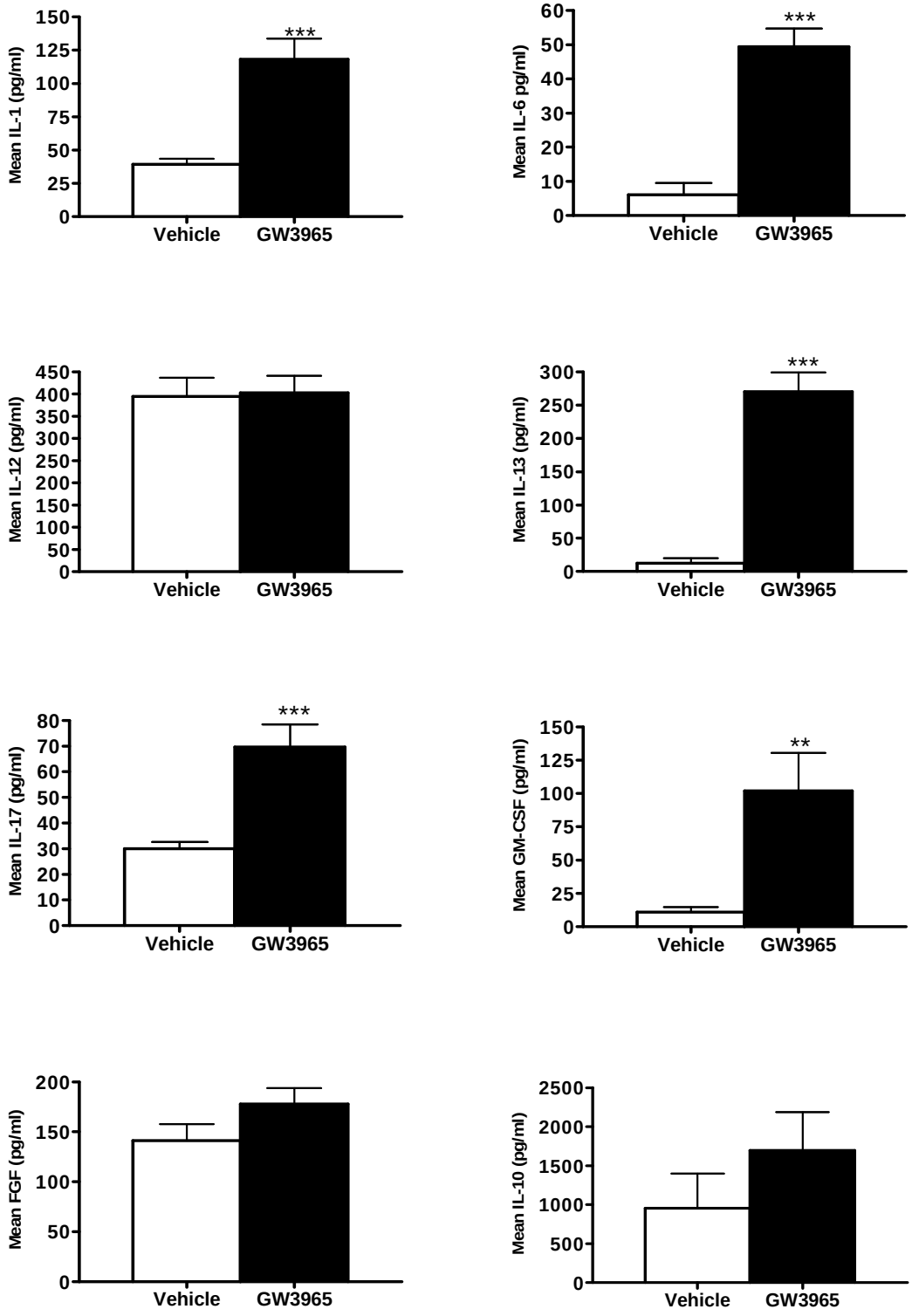


Figure 8.29 GW3965 increases the concentration of serum proinflammatory cytokines. Blood samples were removed from mice at day 31 of the CIA model by cardiac puncture and the concentration of serum cytokines was measured by Luminex. Student T test; * $P \leq 0.05$, *** $P \leq 0.001$. n =12/ group.

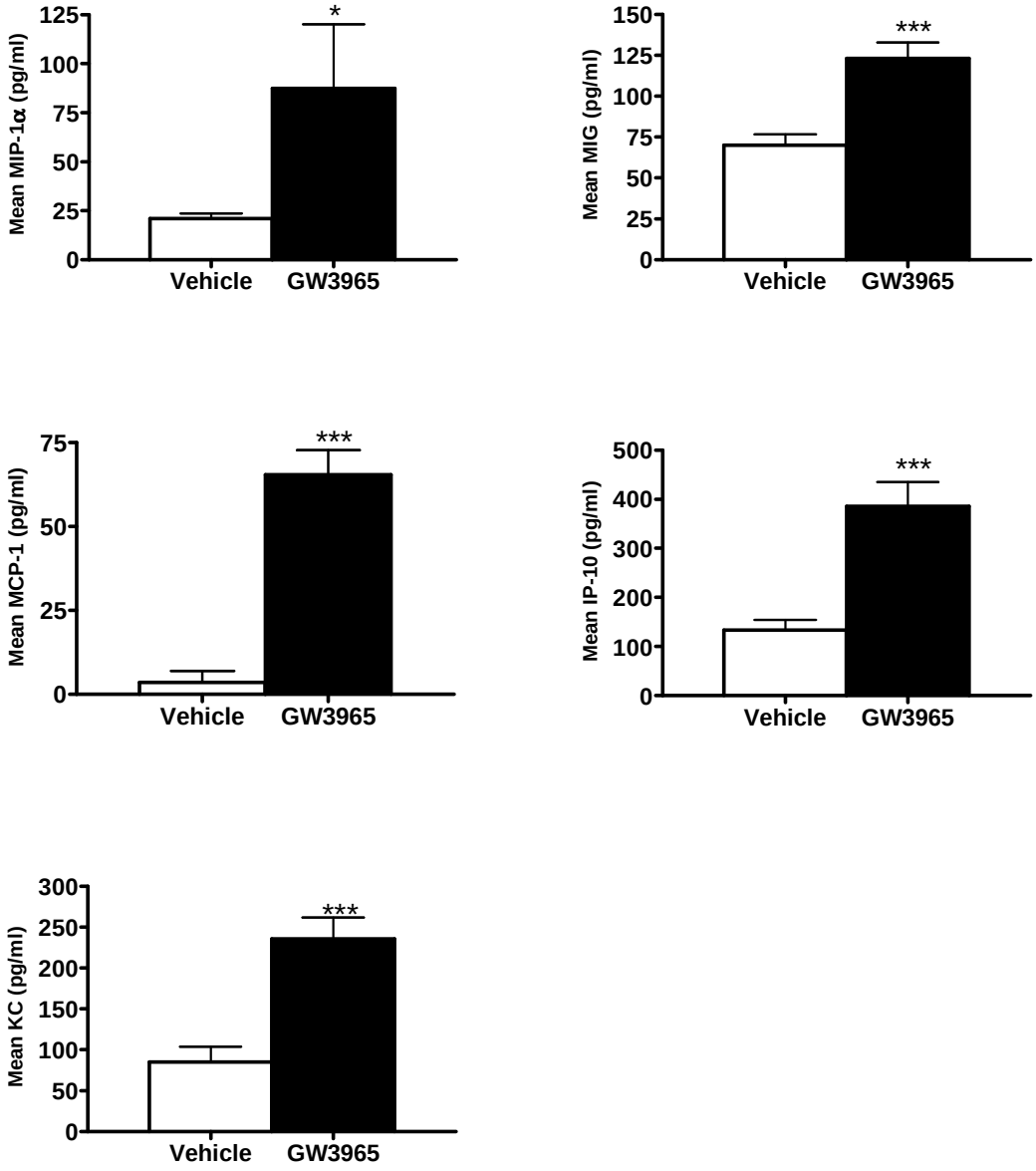


Figure 8.30 GW3965 increases the concentration of serum chemokines.
The concentrations of multiple pro-inflammatory chemokines are increased in mice receiving 30 mg/kg GW3965 relative to vehicle as measured by Luminex analysis. Other inflammatory chemokines were below the level of detection. Unpaired Students T test; * P ≤ 0.05, *** P ≤ 0.001. n = 12/group.

To determine the effect of GW3965 upon T cell activation the popliteal and inguinal lymph nodes were removed and crushed through a cell strainer to generate a single cell suspension. The cells were stimulated overnight with PMA and Ionomycin and the percentage of $CD4^+$ cells that were $IFN\gamma^+$ (Th1) or $IL\text{-}17^+$ (Th17) was analysed by flow cytometry (Figure 8.31). The percentage of Th1 ($CD4^+$, $IFN\gamma^+$) and Th17 ($CD4^+$, $IL\text{-}17^+$) cells is higher in mice treated with GW3965 relative to vehicle. However, the most striking difference was in the $CD4^-$

population as there was a significant increase in the percentage of IL-17$^+$ or IFNγ$^+$ cells; the identity of which are unknown.

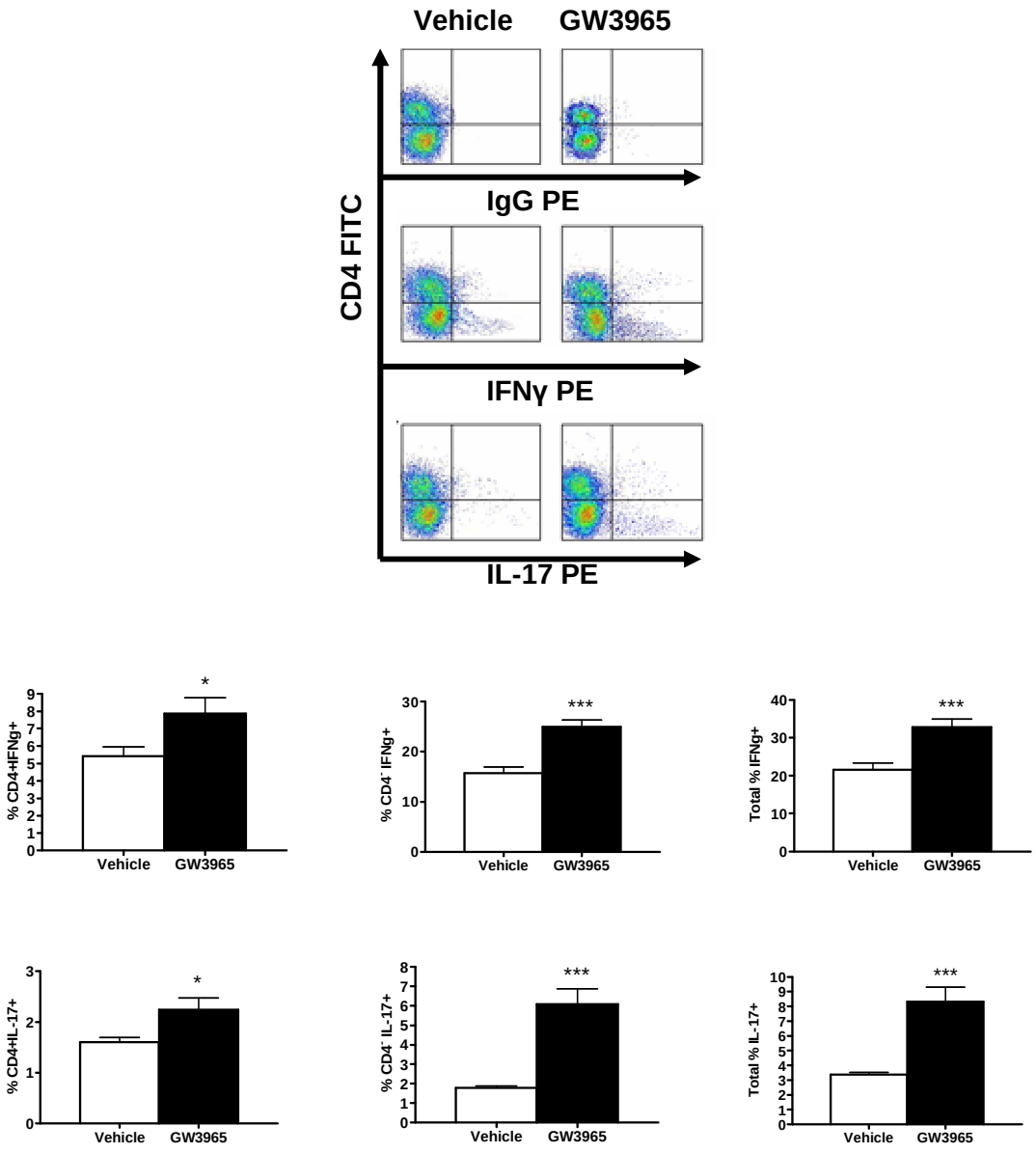


Figure 8.31 Increased activation of lymph node cells by GW3965.
The percentage of Th1 (CD4$^+$, IFNγ$^+$) and Th17 (CD4$^+$, IL-17$^+$) cells is higher in mice treated with GW3965 relative to vehicle (5% mulgofen/ PBS). There is also a significant increase in the percentage of CD4$^-$, IL-17$^+$ or IFNγ$^+$. The identity of these cells is unknown. Unpaired Students T test; * P ≤ 0.05, ** P ≤ 0.001. n = 12/group.

The concentration of serum anti-collagen antibodies was measured by ELISA. The titers of IgG2$_a$ and IgG1 anti-collagen antibodies were higher in the group receiving 30 mg/kg GW3965 relative to vehicle providing clear evidence of enhanced antigen specific adaptive responses in GW3965 treated mice (Figure 8.32).

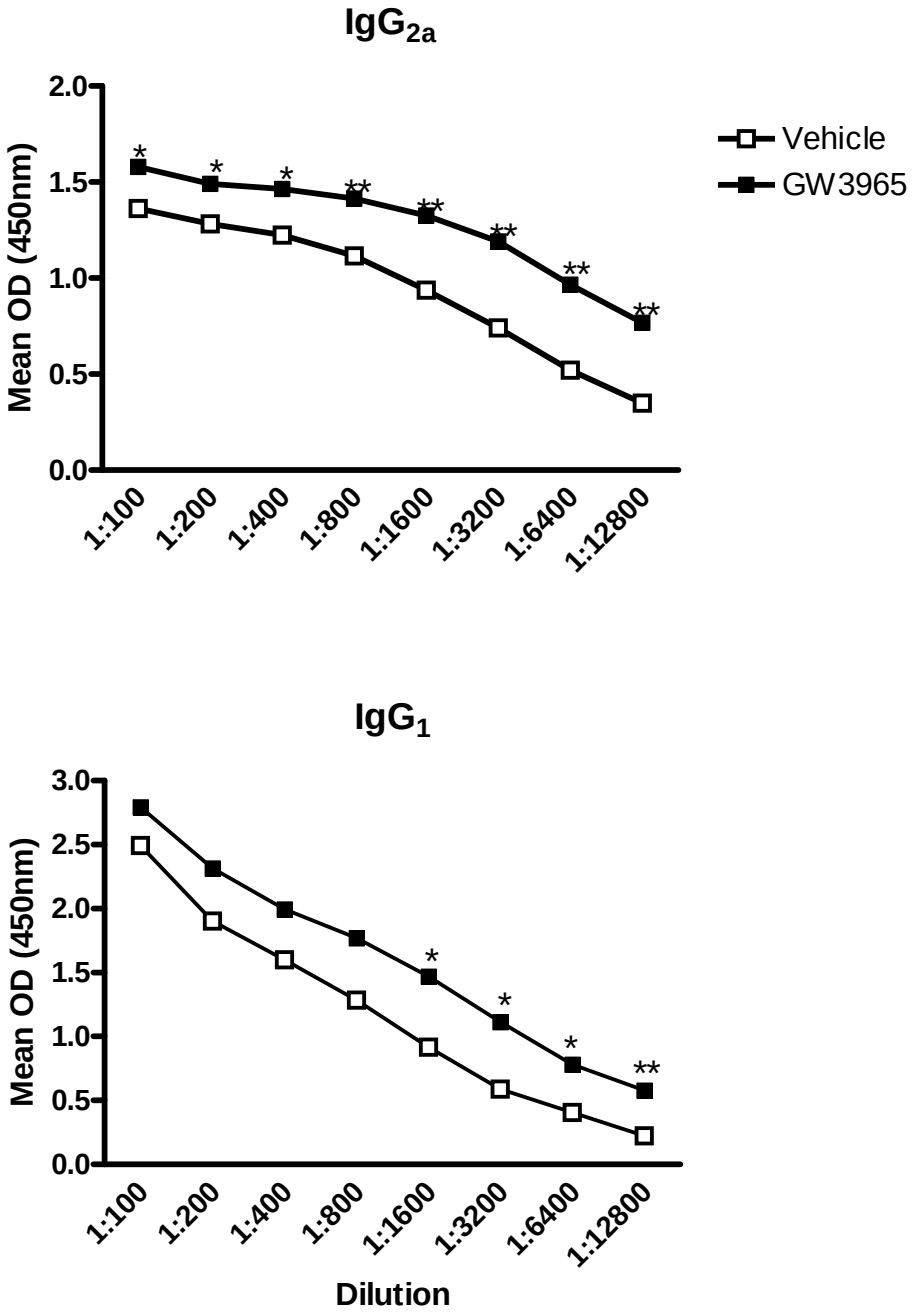


Figure 8.32 Anti-collagen antibody titres are increased by GW3965.
Serially diluted serum analysis of IgG2a and IgG1 anti-collagen antibodies in mice receiving 30 mg/kg GW3965 relative to vehicle (5% mulgofen/ PBS). Unpaired students T test * P ≤ 0.05, ** P ≤ 0.01. n =12/ group.

8.3.3.3 GW3965 induced activation of LXRs

To confirm LXR activation by GW3965 the jejunum was removed six hours post drug administration and the expression of ABCA1 was measured by Taqman QRT-PCR. The expression of ABCA1 was upregulated approximately twenty fold in the

GW3965 group relative to the vehicle control indicating robust transcriptional activation of LXRs (Figure 8.33).

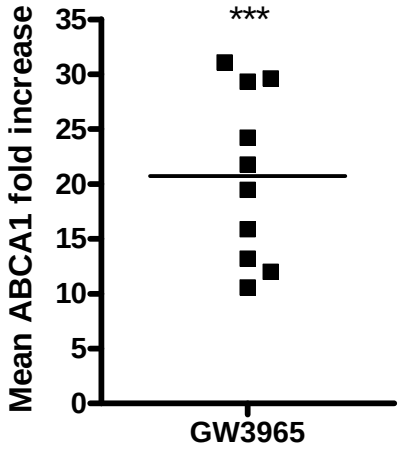


Figure 8.33 Induction of LXR transcriptional activation by GW3965.
The expression of the LXR reporter gene ABCA1 was measured by Taqman QRT-PCR in the jejunum of mice treated with 30 mg/Kg GW3965 or vehicle at the indicated concentration. Results are displayed as mean fold increase of ABCA1 expression relative to vehicle. n = 12/ group. Students T test; *** P ≤ 0.001.

8.4 Discussion & conclusion

Previous studies have suggested a regulatory role for LXRs upon inflammation. Therefore, this study was initiated to determine if LXR agonists could offer beneficial effects upon inflammation in the joint. However, contrary to my expectations, the incidence and severity of arthritis was markedly increased thus suggesting a novel pro-inflammatory role for LXRs.

Preliminary experiments were set up to determine the optimal route of agonist administration. These studies demonstrated that IP injection was a suitable route for delivery of LXR agonists *in vivo* and was optimal for bioavailability LXR agonists in the tested tissues; liver and bone marrow. Early studies also addressed the use of 5% mulgofen/ PBS as a vehicle in which to deliver agonists. These were initiated under the premise that mulgofen may contribute towards the inflammatory aspect of arthritis and could be further amplified by LXR agonism and thereby potentially explaining the unexpected pro-inflammatory effect of LXR agonism. However, comparison of vehicle with PBS showed that there was no effect upon inflammation and the subsequent severity of arthritis. This suggests that any effect upon inflammation and the severity of disease are mediated specifically by drug administration.

Initial experiments analysing the role of LXRs upon inflammation in arthritis were limited by the use of T1317 which increased the severity of disease in a dose responsive manner. However, in addition to LXR, T1317 has also been shown to agonise Pregnane X receptor (PXR) and Farnesoid X receptor (FXR) which primarily regulate carbohydrate, lipid and bile acid metabolism but their role in inflammation is as yet unknown (297, 298). Therefore, any observed effects could be at least in part attributed to non-specific activation of PXR and/ or FXR by T1317. In comparison GW3965 is highly specific and does not exert any *known* off target effects on any other nuclear receptors. Therefore, results obtained using GW3965 probably more accurately reflect the role of LXRs in inflammation. However, administration of 10 mg/Kg GW3965 did not significantly increase the severity of disease. It is possible that the concentration of 10 mg/kg GW3965 used was too low. Indeed, although the results were significant, 10 mg/kg T1317 had only a small effect upon serum cytokine and

chemokine concentrations (Figure 8.19). This is consistent with *in vitro* findings where the effect of GW3965 upon cytokine secretion is generally lower than that mediated by T1317(Figure 10.18). Furthermore, treatment of mice at a higher concentration, with 30 mg/Kg GW3965, significantly increased the severity of CIA. Although the use of these highly selective agonists suggest a specific pro-inflammatory effect off target effects of LXR agonism can not be precluded and will be dealt with in subsequent studies in LXR deficient mice.

The severity of disease was also increased by a longer time course of T1317 administration (Figure 8.10). It is unlikely that this reflects accumulation of T1317 over a prolonged time course as the expression of ABCA1 was comparable between the early and late time courses of agonist administration; suggesting that the level of LXR activation, and therefore the tissue specific concentration of agonist, was similar between the two treatment groups. These results are therefore more likely to reflect the prolonged action of LXRs upon inflammatory pathways.

We hypothesised that LXR agonism would be therapeutic in CIA. Mice were therefore treated with drugs up to day 42 as it was unknown in which phase of disease LXR agonists would have an effect i.e. the priming or resolution phase of disease. However, LXR agonists dramatically increased the severity of disease and as such individuals had to be euthanised at multiple time points to comply with Home Office regulations. This therefore introduced a large degree of variability making it difficult to draw firm conclusions from my first *ex vivo* analysis. This is exemplified in the groups treated with 20 mg/Kg T1317 in which although clinical signs of disease were markedly increased, i.e. clinical score and paw thickness, there was little or no effect upon serum cytokine/chemokine concentrations, lymphocyte activation and anti-collagen antibody titres. CIA in the DBA/1 mouse has been well characterised - the severity of disease peaks at approximately day 34 after which the anti-inflammatory cytokines IL-10 and TGF-β are upregulated leading to inhibition of inflammation and resolution of disease. It was therefore necessary to terminate the experiment at an earlier time point in subsequent studies in order to accurately analyse the effect of LXR agonism upon pro-inflammatory mediators at the peak of disease. As such in later experiments, C57BL/6 mice were treated with 30 mg/Kg of GW3965 up to day 31 post induction of arthritis in which the level of proinflammatory

cytokines/ chemokines, lymphocyte activation and anti-collagen antibody titres were clearly elevated compared to controls.

Together these data support a pro-inflammatory role for LXRs however the mechanism whereby LXR agonists promote articular inflammation *in vivo* is not clear. The numbers of activated lymphocytes were increased by LXR agonism characterised by an increased number of IL-17 and IFNγ secreting cells suggesting amplification of effector T cell subsets, namely Th1 and Th17 cells. Interestingly, there was also a substantial increase in the proportion of $CD4^-$ IFNγ and IL-17 positive cells which likely comprise multiple cell types including NK cells, γδ T cells, $CD8^+$ T cells and macrophages (174, 175). Since the effect of LXR agonism is not limited to a specific cell type it is possible that the amplification of a pro-inflammatory cytokine milieu *in vivo* is capable of supporting T cell differentiation, characterized by increased serum concentrations of IL-6, IL-12 and IL-23. Additionally, the increased concentration of anti-collagen specific antibodies further suggests that LXR agonism was able to exert a general, but context specific, pro-inflammatory effect and thereby enhance both the innate and adaptive immune responses. Further, studies will therefore be required to identify the affects of LXR agonism on the activation and differentiation of specific inflammatory cell types.

Both T1317 and GW3965 are dual LXR agonists and therefore induce the activation of both LXRα and LXRß. The individual role of LXRα and LXRß is currently being studied in the context of atherosclerosis in which it is evident that agonists specifically targeting LXRß may be more favourable for ameliorating atherosclerotic lesions (37, 101, 102, 299). LXR agonism has been shown to result in hepatic steatosis in the $ApoE^{-/-}$ model of atherosclerosis. It is therefore possible that hepatic steatosis could operate as a potential driver of acute phase responses and hence inflammation and thereby enhance disease severity. Indeed, in preliminary experiments in which mice were treated for seven days with T1317 the expression of FAS, the rate limiting enzyme involved in lipogenesis, was significantly upregulated which has been shown to lead to the accumulation of triglycerides within the liver (Figure 8.3). The effect of prolonged agonist treatment in the CIA model upon hepatic FAS expression and lipid accumulation was not tested; this will be addressed in ongoing studies using LXR knockout animals. The effect of LXR agonism upon driving hepatic steatosis

is lost in ApoE$^{-/-}$, LXRα$^{-/-}$ double knockout mice. It is now well accepted that hepatic steatosis by LXR agonism is primarily driven by LXRα. Therefore, as discrete functions are emerging in metabolism, it is also possible that LXRα or LXRß may have different, and potentially opposing, roles in inflammation. Further studies are required to identify the individual role of LXRα and LXRß on immune pathways.

The data obtained from these experiments contradict the work by Chintalacharuvu *et al*, who reported that treatment of established CIA with T1317 yielded beneficial effects; i.e. reduced disease severity (125). However, there are differences between the two studies. Firstly, Chintalacharuvu *et al* used oral gavage for administration of T1317 whereas we administered all drugs IP. Interestingly my preliminary experiments demonstrated that, in comparison to IP, oral gavage of T1317 did not induce the expression of ABCA1 in the bone marrow suggesting restricted bioavailability of agonist when administered by oral gavage. Furthermore, administration of dexamethasone was suppressive showing that there were no unexpected adverse effects of multiple IP injections. Secondly, the treatment protocols and drug dose are not identical. In other studies, T1317 was used at 50 mg/Kg over a much shorter time course, day 27 to day 33 post induction of arthritis, with no evidence of dose dependent effects at lower agonist concentrations. It is therefore possible that T1317 when used at such high concentrations may exert non-specific effects promoting anti-inflammatory pathways. In addition, these data were not reproduced using the highly specific LXR agonist GW3965 or supported by data from LXR deficient animals. The precise effects of T1317 upon LXR activation at such high concentrations has not previously been reported in the literature. Chintalacharuvu *et al* did not document LXR activation in their model and it is therefore possible that increasing concentrations of T1317 may exert unrecognised bi-phasic effects upon the activation of LXRs.

In conclusion, the precise mechanism in which LXRs are able to augment articular inflammation is not clear. However, my data suggest that the mechanism *in vivo* is in part through the ability to exacerbate the secretion of proinflammatory chemokines and cytokines and thereby enhance the recruitment and activation of inflammatory cells to the site of inflammation. In

subsequent chapters I shall describe experiments designed to further explore these possibilities.

9 Exacerbation of articular Inflammation is specific to the activation of Liver X Receptors

9.1 Introduction & Aim

Several studies have suggested a novel role for LXRs in inflammation in which pharmacological activation of LXRs was generally ascribed an anti-inflammatory effect (19, 104, 117, 121). These initial studies demonstrated that activation of LXRs would be beneficial for the treatment of human inflammatory disorders. I therefore previously sought to determine the effect of LXR activation upon inflammation in RA by utilising the murine model of CIA. However, contrary to my expectations and other observations in the literature (125), LXR agonism exerted potent pro-inflammatory effects and markedly accelerated the time of onset and severity of arthritis. In an attempt to prove that the pro-inflammatory effect was mediated specifically through the activation of LXRs two pharmacologically distinct LXR agonists were used; GW3965 and T1317. Both compounds are widely used LXRs agonists; however, whilst both agonists demonstrate similar efficacy in murine models of atherosclerosis it is well recognised that T1317 activates the nuclear receptors FXR and PXR non-specifically (297, 298). Moreover, whereas GW3965 is considered to be highly specific and does not induce the activation of other nuclear receptors, off target effects of GW3965 cannot be discounted as a potential explanation for the unexpected pro-inflammatory effects observed *in vivo*.

CIA has been widely used as a murine model of arthritis in which susceptibility is restricted to strains, such as DBA/1 mice, with the MHC Class II I-A^q haplotype (222). Most transgenic mice, including currently available *LXRa*$^{-/-}$ and *LXRB*$^{-/-}$ mice, are on the C57BL/6 background which is generally considered resistant to CIA. However, with the recent development of a refined protocol it is now possible to induce CIA in C57BL/6 mice and therefore study the effect of LXR agonist administration in LXR deficient mice (225). *I therefore sought to determine if the pro-inflammatory effects of LXR agonists in the murine collagen-induced arthritis model was mediated specifically by the activation of LXRs.*

9.2 Induction of CIA in C57BL/6 mice

It was first necessary to ensure that CIA could be induced in the C57BL/6 strain. Following the protocol by Inglis *et al,* arthritis was induced in male wild-type C57BL/6 mice at 10 weeks of age by inoculation with 200 µg of type II chicken collagen/ CFA injected intradermally at the base of the tail (225). Three weeks later (day 21) mice were challenged with 200 µg type II collagen/ PBS injected IP and monitored for clinical signs of disease. Arthritis was evident from day 21 onwards and consistent with the findings of Inglis *et al* 50% of the animals had developed clinical signs of arthritis by day 40 (Figure 9.1). The severity of disease was also monitored and increased from day 21 onwards. However, unlike arthritis in the DBA/1 mice that is self remitting the severity of disease in the C57BL/6 mice was maintained for up to ten weeks post induction of arthritis (Figure 9.2). These experiments provided evidence that arthritis could successfully be induced in the C57BL/6 strain of mice by this protocol.

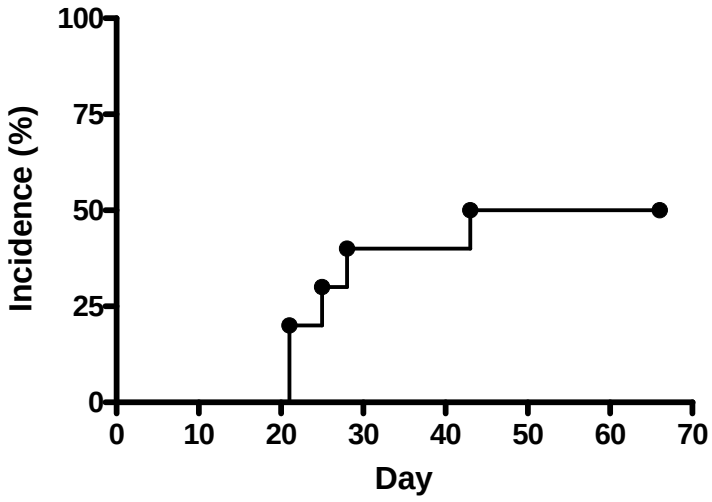


Figure 9.1 Incidence of arthritis in wild-type C57BL/6 mice.
Arthritis was induced in male C57BL/6 mice at 10 weeks of age following the published protocol by Inglis *et al* (225). Mice were monitored from day 21 onwards for clinical signs of disease. n = 10.

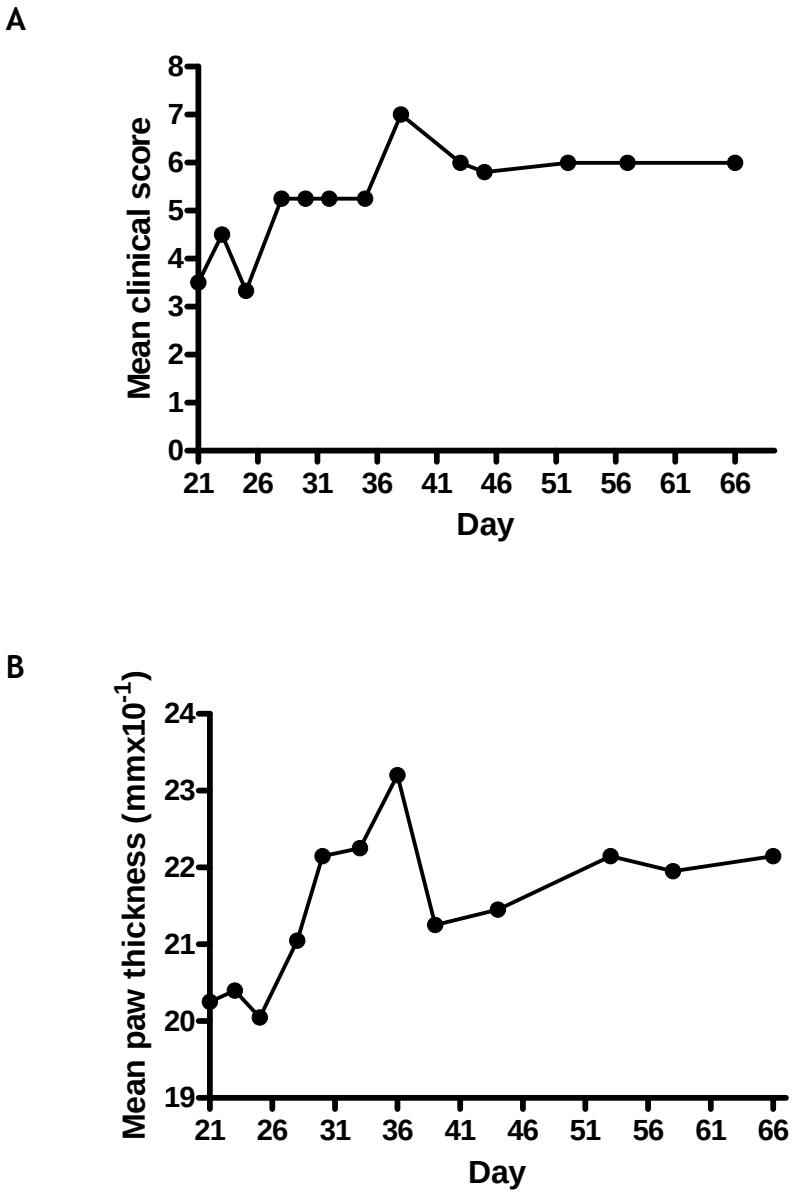
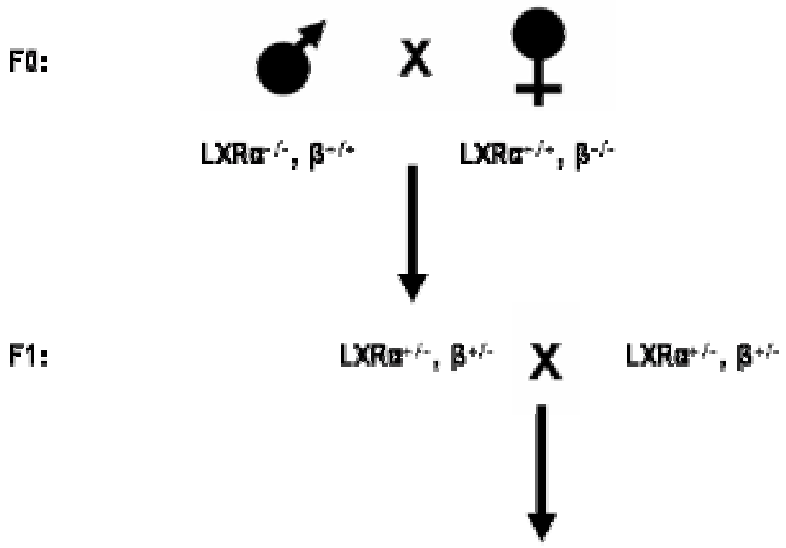


Figure 9.2 Chronic arthritis in C57BL/6 mice.
The severity of arthritis in C57BL/6 mice was monitored from day 21 onwards. Mice were assigned a clinical score (A) whilst paw swelling was measured as an independent measure of disease severity (B). The severity of disease was maintained for up to 10 weeks at which point the experiment was terminated. n = 10.

9.3 Generation of LXR double KO mice

LXRα$^{-/-}$ and *LXRβ*$^{-/-}$ (single knockouts) have been generated to study the role of LXRs in atherosclerosis however, the effect of LXR deficiency and the role of the individual LXR isoforms in the context of inflammation is unknown (101, 102). Deletion of LXRα or LXRβ is associated with increased susceptibility to atherosclerotic plaque formation and enhanced autoimmunity, this is in part mediated through the inability to clear apoptotic thymocytes and B cells (300). Both LXRα and LXRβ share some of the same target genes; e.g. ABCA1/ G1, LDLR

and NPC-1 (49, 83-85), which suggests that deficiency of one isoform may be potentially compensated by the other. Additionally, T1317 and GW3965 are dual LXRα/ LXRβ agonists therefore the pro-inflammatory effect of LXR agonism may be mediated by either LXRα or LXRβ. Therefore, to further investigate the individual role for LXRα and LXRβ and demonstrate an LXR specific pro-inflammatory effect of GW3965 administration in arthritis it was necessary to generate LXRα/β double KO mice (*LXRα/β KO*). A schematic of the breeding programme is illustrated in Figure 9.3. $LXR\alpha^{-/-}$ and $LXR\beta^{-/-}$ mice, supplied by Schering Plough, were crossed to generate LXRα/β heterozygotes ($LXR\alpha^{+/-}$, $LXR\beta^{+/-}$) (F1 generation); this was confirmed by PCR screening of the LXRβ genotype of genomic DNA (Figure 9.4). Seven breeding pairs from the F1 were set up and self-crossed to generate the F2 offspring which were all screened by PCR for the LXRα and LXRβ genotype. All the potential genotypes are illustrated in Figure 9.3 and the chance of generating an *LXRα/β KO* mouse, of either sex, is 1:16. Three *LXRα/β KO* mice were generated; two females #15 and #24 and one male #22 (Figure 9.5). From these three mice female #15 and male #24 were selected for further breeding to generate more breeding pairs (F3 generation) to setup an *LXRα/β KO* breeding colony. The genotype of the F3 was confirmed by PCR screening to be *LXRα/β KO* before further breeding was allowed (Figure 9.6).



F2:

Genotype	α^{+},β^{+}	α^{+},β^{-}	α^{-},β^{+}	α^{-},β^{-}
α^{+},β^{+}	$\alpha^{+/+},\beta^{+/+}$	$\alpha^{+/+},\beta^{+/-}$	$\alpha^{+/-},\beta^{+/+}$	$\alpha^{+/-},\beta^{+/-}$
α^{+},β^{-}	$\alpha^{+/+},\beta^{-/-}$	$\alpha^{+/+},\beta^{-/-}$	$\alpha^{+/-},\beta^{+/-}$	$\alpha^{+/-},\beta^{-/-}$
α^{-},β^{+}	$\alpha^{+/-},\beta^{+/+}$	$\alpha^{+/-},\beta^{+/-}$	$\alpha^{-/-},\beta^{+/+}$	$\alpha^{-/-},\beta^{+/-}$
α^{-},β^{-}	$\alpha^{+/-},\beta^{+/-}$	$\alpha^{+/-},\beta^{-/-}$	$\alpha^{-/-},\beta^{+/-}$	$\alpha^{-/-},\beta^{-/-}$

Figure 9.3 Generation of the LXR alpha/beta double knockout mice
A schematic showing generation of the *LXRα/β KO* mice. *LXRα*$^{-/-}$ mice were crossed with *LXRβ*$^{-/-}$ mice to generate LXR α/β heterozygotes (F1). The F1 generation was then self crossed (F1 X F1) to generate the LXR double KO mice (F2). The table demonstrates all the potential genotypes that can arise from the F1 cross and giving a 1:16 chance of generating an *LXRα/β* double knockout mouse of either sex.

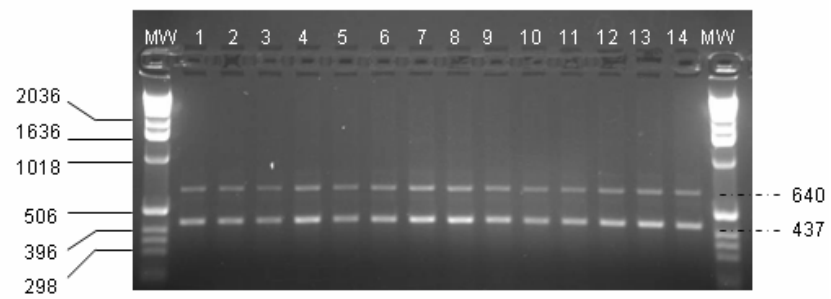


Figure 9.4 Generation of LXR heterozygotes: F1 generation.
Tail tips from potential *LXRα/β* heterozygotes were digested and the genomic DNA extracted (see materials & methods). A PCR of genomic DNA for LXRβ was used to confirm the genotype which was visualised on a 2% agarose gel; LXRβ KO ~640 bp and LXRβ WT ~ 437bp. Molecular weight ladder (MW) and samples 1 to 14.

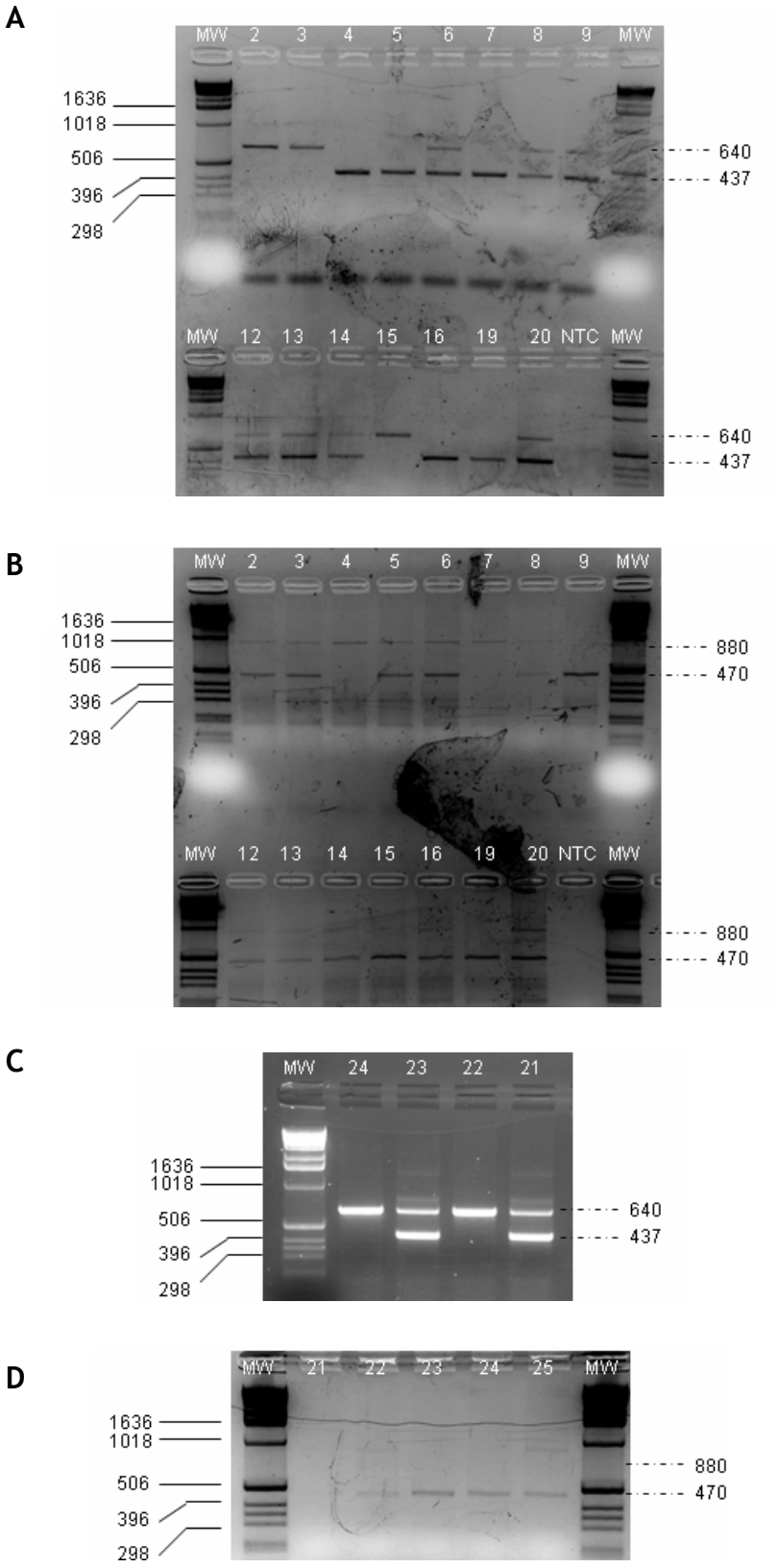


Figure 9.5 Generation of *LXRα/β* double KO mice: F2 generation.
Tail tips from potential *LXRα/β* double KO were digested and the genomic DNA extracted (see materials & methods). A PCR of genomic DNA for LXRβ (A, C) and LXRα (B, D) was used to confirm the genotype which was visualised on a 2% agarose gel against a molecular weight ladder (MW). LXRβ KO ~640 bp and LXRβ WT ~437bp, LXRα KO ~470 bp and LXRα WT ~880 bp. Number 15, 24 and 22 were LXRα/β KO; the later was the only male.

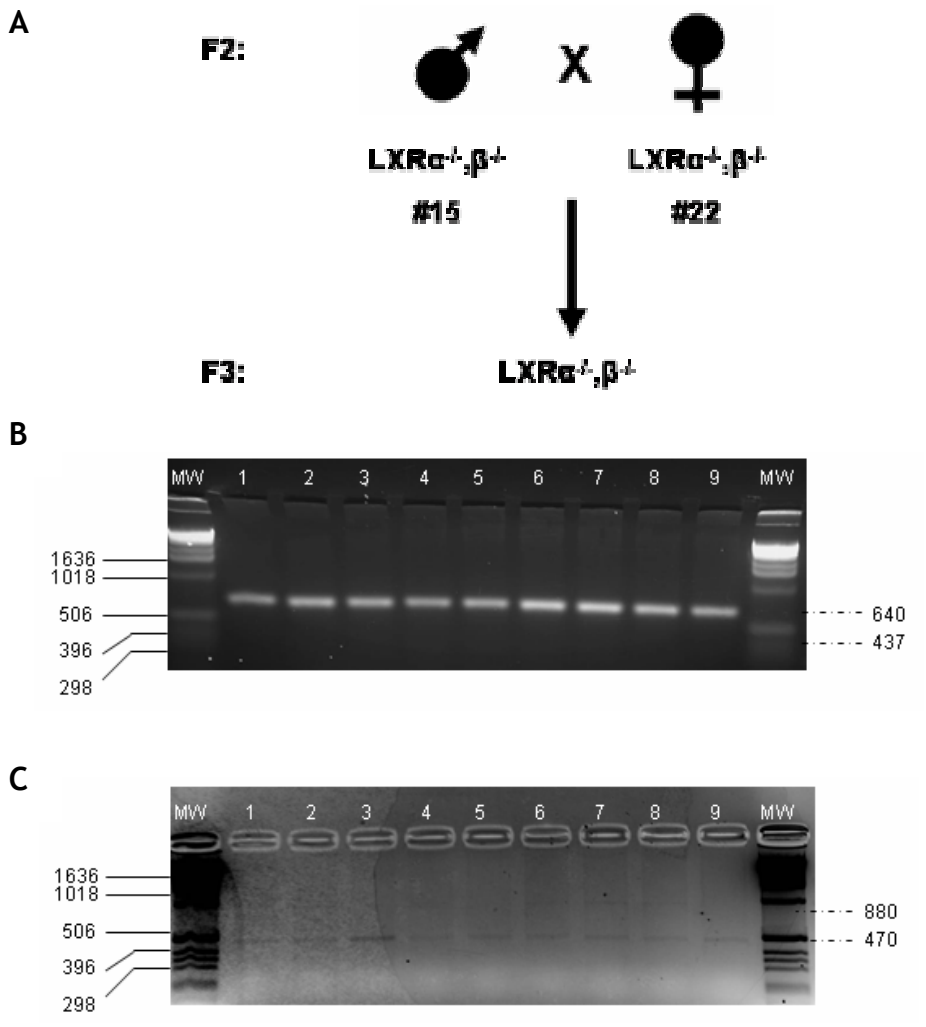


Figure 9.6 Confirmation of LXRα/β double knockout genotype in F3 offspring.
(A) Schematic showing that LXRα/β KO mice (F2) were self crossed to generate breeding pairs for an LXRα/β KO breeding colony (F3). PCR screening of genomic DNA for LXRβ (B) LXRα (C) was visualised on a 2% agarose gel against a molecular weight ladder (MW). LXRβ KO ~640 bp and LXRβ WT ~437 bp, LXRα KO ~470 bp and LXRα WT ~880 bp.

9.4 Induction of CIA in LXR deficient mice

9.4.1 The effect of GW3965 in arthritis is mediated specifically by activation of LXRs

With the successful generation of the LXRα/β KO mice and confirmation that it is possible to induce CIA in the C57BL/6 strain of mice it was possible to proceed and determine if the pro-inflammatory effects of GW3965 in arthritis were mediated specifically by agonism of LXRs. Arthritis was induced in male wild-type, *LXRα*$^{-/-}$, *LXRβ*$^{-/-}$ or *LXRα/β KO* mice at approximately 8-11 weeks of age following the protocol described by Inglis *et al* (225). GW3965 is a highly specific LXR agonist and as a dose of 30 mg/Kg was previously used with the greatest pro-inflammatory effect in arthritis this dosing regime was taken forward for future studies. Mice were therefore treated with 30 mg/Kg GW3965 or vehicle (5% mulgofen/ PBS) from day -1 to day 37. A schematic of the experimental design is described in Figure 9.7 and the number of mice in each group is shown in Table 9.1. The results of the study are pooled from three independent experiments in which each genotype had equal numbers of mice treated with GW3965 as with vehicle in the same experiment. The mice were monitored daily from the start of the experiment for the development of clinical arthritis. The incidence of arthritis was comparable between all the genotypes and did not differ between the groups treated with vehicle or GW3965 (Figure 9.8); these data are also tabulated in Table 9.1.

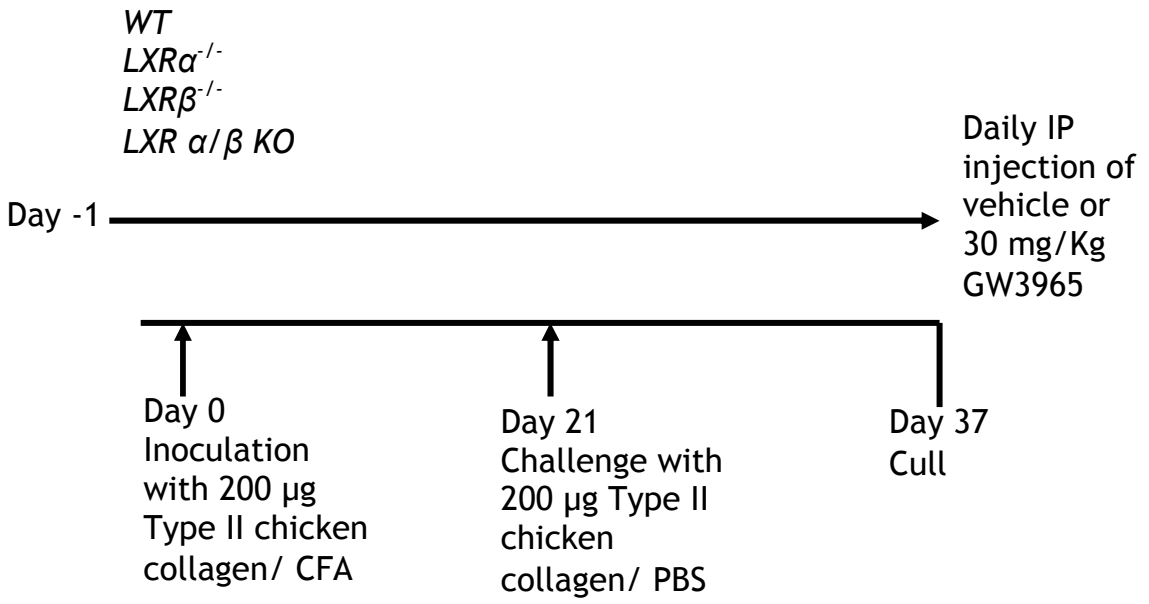


Figure 9.7 Experimental design for the induction of arthritis in LXR deficient mice.

Treatment Group	**Vehicle**		**30 mg/Kg GW3965**	
Genotype	Subjects	Incident (%)	Subjects	Incident (%)
WT	19	10 (52)	18	9 (50)
LXRα$^{-/-}$	27	13 (48)	26	12 (46)
LXRß$^{-/-}$	18	9 (50)	24	10 (42)
LXRα/ß KO	9	5 (55)	10	5 (50)

Table 9.1 The incidence of arthritis in wild-type and LXR deficient mice.
Arthritis was induced in male mice at approximately 8-11 weeks of age on the C57BL/6 background; either wild-type (*WT*), *LXRα*$^{-/-}$, *LXRβ*$^{-/-}$ or *LXRα/β KO*. Mice were treated daily with vehicle (5% mulgofen/PBS) or 30 mg/Kg GW3965 by IP injection. Mice were monitored daily for the first signs of clinical arthritis. The number of mice that were entered into the study (subjects) and the number (and percentage) of incident mice are described for each treatment group of each genotype. Results are pooled from three independent experiments.

The development of arthritis was monitored from day 21 onwards and mice were assigned a clinical score based upon the severity of arthritis as before and described in materials & methods (Figure 9.9). In support of a pro-inflammatory effect of LXR agonism the severity of disease was significantly increased in wild-

type mice treated with GW3965 relative to vehicle. This was specific to wild-type mice as there was no significant difference in the severity of disease in *LXRa*$^{-/-}$ (P = 0.09), *LXRβ*$^{-/-}$ (P = 0.73) or *LXRa/β KO* (P = 0.62) treated with 30 mg/Kg GW3965 compared to vehicle. Similarly, the extent of paw swelling was measured as an independent measure of disease severity which was significantly increased in wild-type mice receiving GW3965 compared to vehicle (Figure 9.10). Consistent with the clinical score there was no significant difference in the paw swelling of *LXRa*$^{-/-}$ (P = 0.3017), *LXRβ*$^{-/-}$ (P = 0.671) or *LXRa/β KO* (P = 0.063) mice treated with GW3965 relative to vehicle controls.

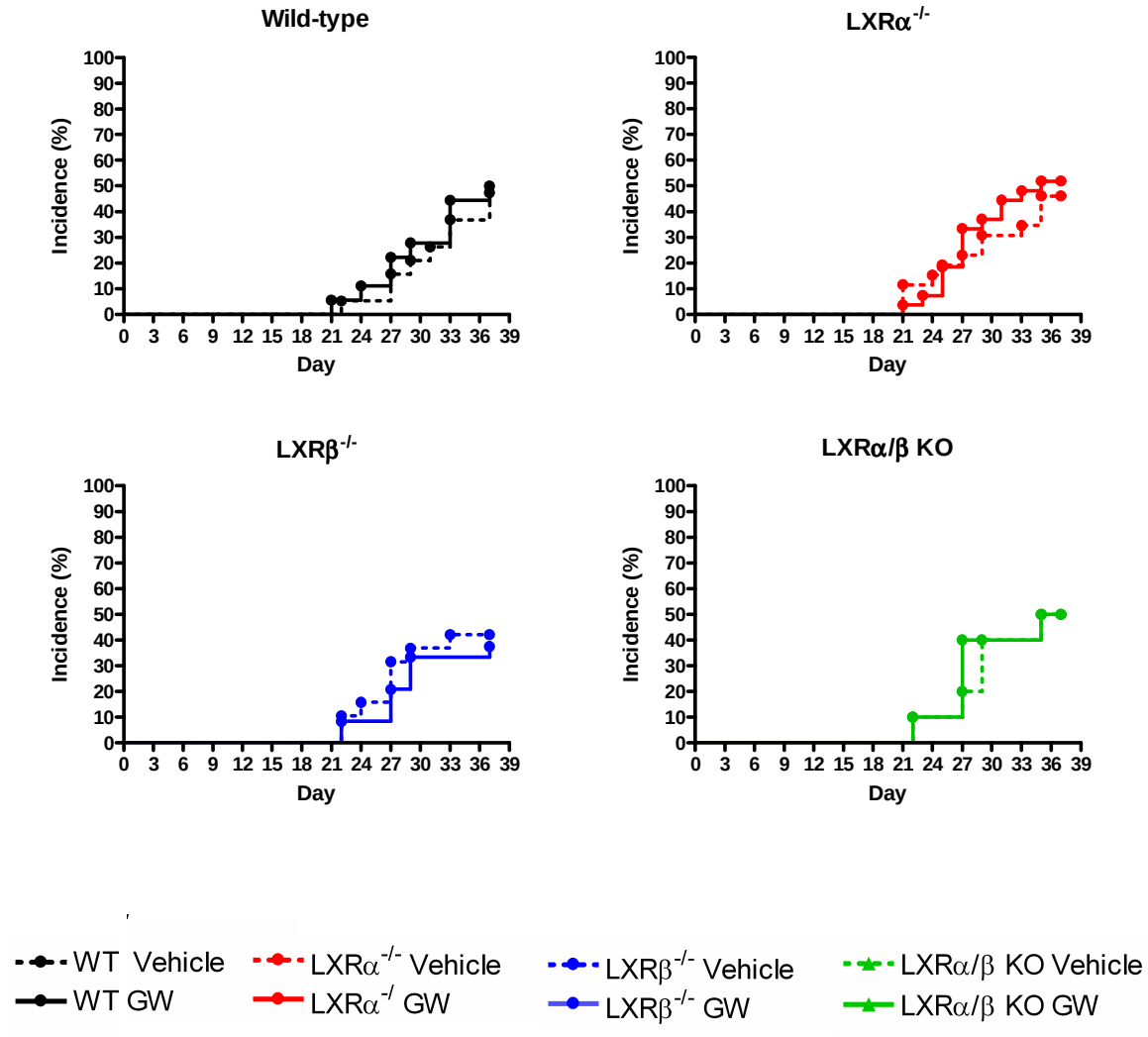


Figure 9.8 The incidence of arthritis in LXR deficient mice.
Arthritis was induced in male mice at approximately 8-11 weeks of age on the C57BL/6 background; either wild-type (*WT*), *LXRα*$^{-/-}$, *LXRβ*$^{-/-}$ or *LXRα/β KO*. Mice were treated daily with vehicle (5% mulgofen/PBS) or 30 mg/Kg GW3965 by IP injection. Mice were monitored daily from day 21 onwards for the first signs of clinical arthritis. Results are pooled from three independent experiments and are also tabulated in Table 9.1.

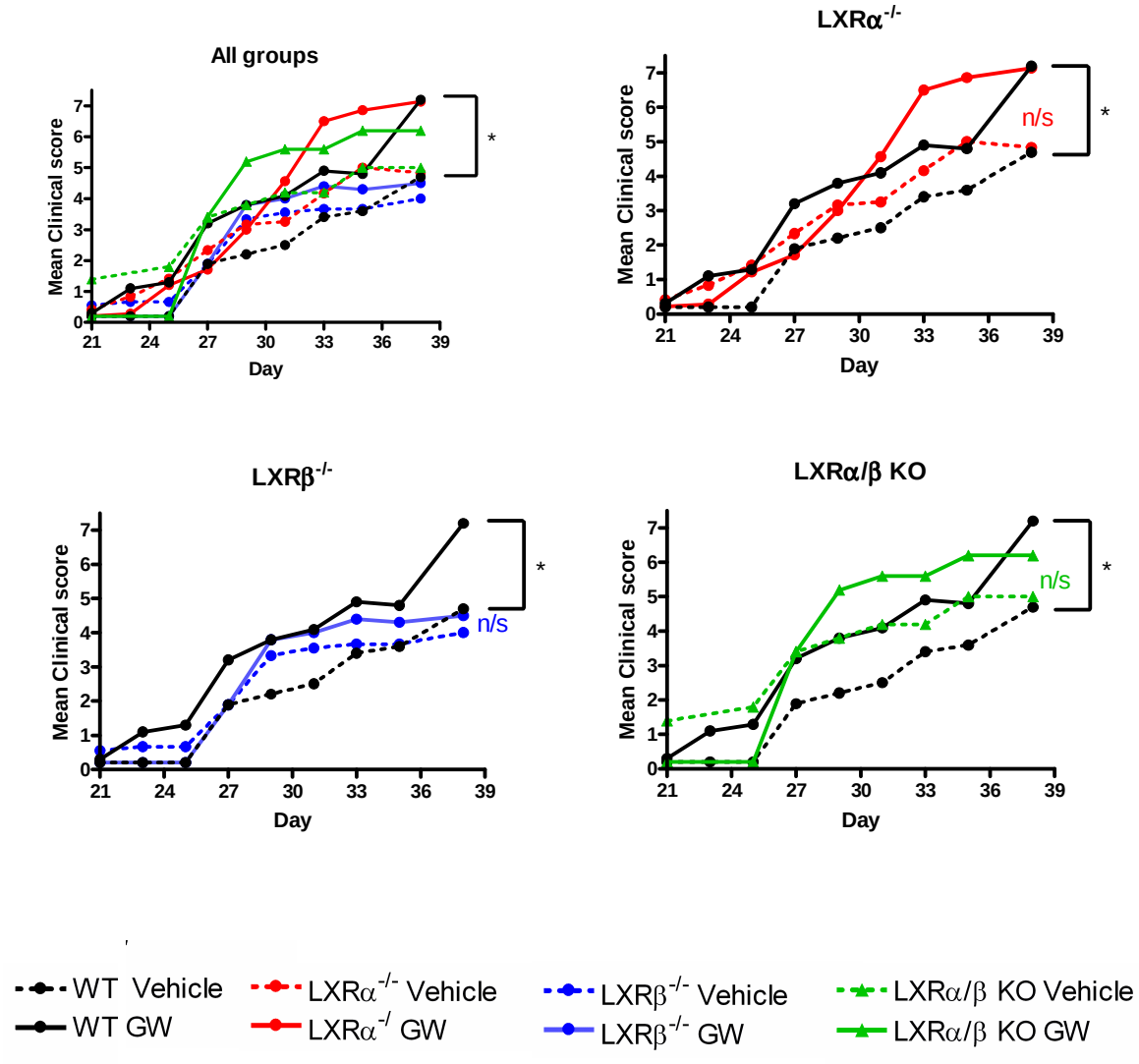


Figure 9.9 Increased severity of clinical arthritis is mediated specifically by LXRs. Arthritis was induced in male mice at approximately 8-11 weeks of age on the C57BL/6 background; either wild-type (*WT*), *LXRα*$^{-/-}$, *LXRβ*$^{-/-}$ or *LXRα/β KO*. Mice were treated daily with vehicle (5% mulgofen/PBS) (dotted lines) or 30 mg/Kg GW3965 (solid lines) by IP injection. Mice were monitored daily and assigned a clinical score as a measure of arthritis severity. Results are pooled from three independent experiments. The number of contributing animals is shown in Table 9.1. Two way ANOVA; * P $\leq$ 0.05, not significant (n/s), with respect to GW3965 vs. vehicle within the same genotype.

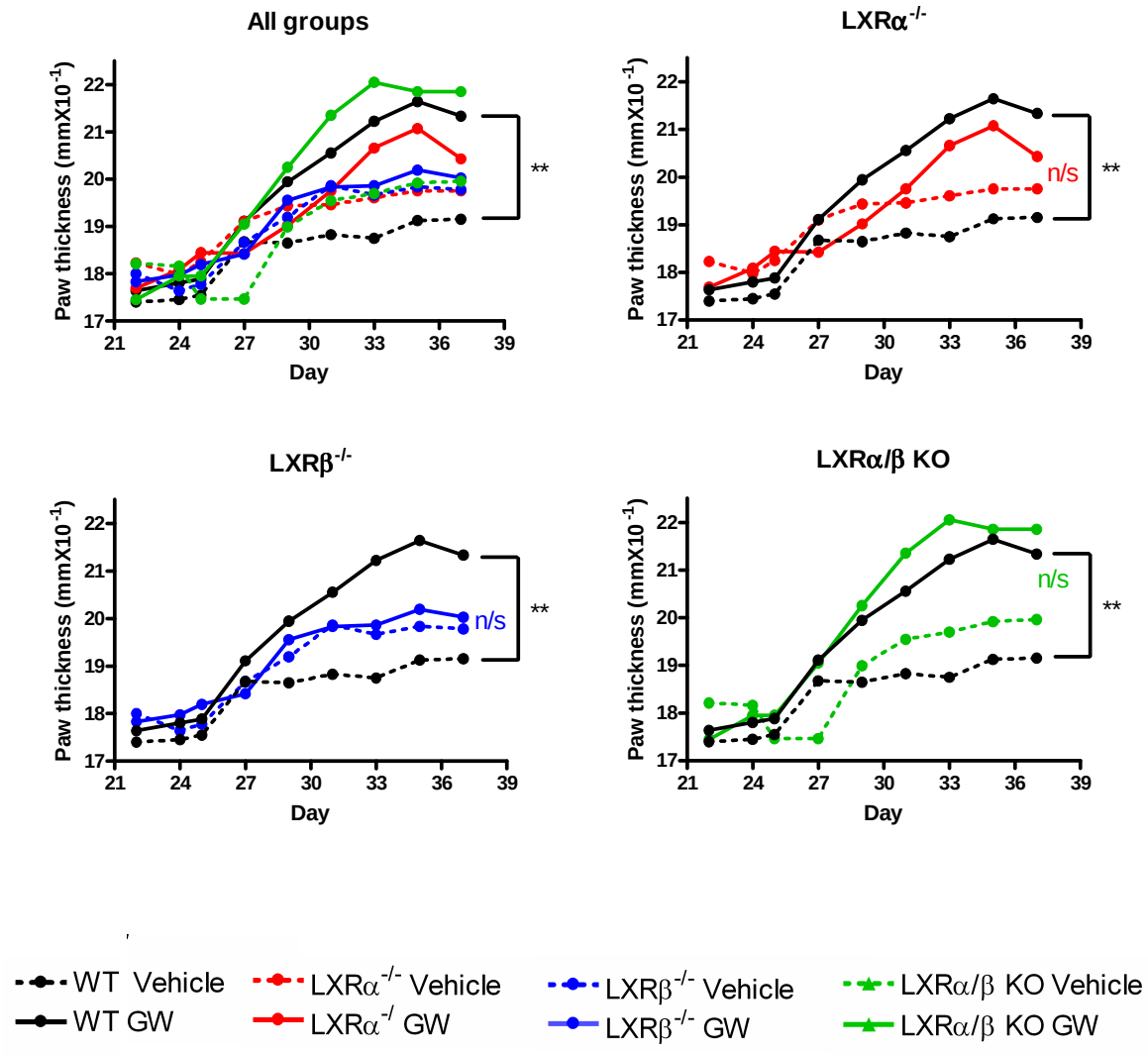


Figure 9.10 Increased paw swelling is mediated specifically by LXRs.
Arthritis was induced in male mice at approximately 8-11 weeks of age on the C57BL/6 background; either wild-type (*WT*), *LXRα*$^{-/-}$, *LXRβ*$^{-/-}$ or *LXRα/β KO*. Mice were treated daily with vehicle (5% mulgofen/PBS) (dotted lines) or 30 mg/Kg GW3965 (solid lines) by IP injection. Mice were monitored daily and paw thickness was measured as an independent measure of arthritis severity. Results are pooled from three independent experiments. The number of contributing animals is shown in Table 9.1. Two way ANOVA; ** P ≤ 0.01, not significant (n/s), with respect to GW3965 vs. vehicle within the same genotype.

9.4.2 The induction ABCA1 expression is diminished in LXR deficient mice

To confirm LXR activation, and loss of LXR induced transcription in LXR deficient mice, the jejunum was removed from mice at day 37 and snap frozen for RNA extraction. Nine samples were chosen at random representative of each group and the expression of ABCA1 was measured by Taqman QRT-PCR (Figure 9.11). Similar to previous results treatment of wild-type mice with 30 mg/Kg GW3965 induced an approximate 15 fold increase in the expression of ABCA1 (compare to Figure 9.11 to Figure 8.33) . As expected the expression of ABCA1 was also significantly increased in *LXRa*$^{-/-}$ mice treated with GW3965 and to a lesser extent in *LXRB*$^{-/-}$ by approximately eight and four fold respectively (37, 101, 102). The expression of ABCA1 in *LXRa/β KO* mice was not significantly different between the vehicle and GW3965 treated group (P = 0.235); however, the basal level of ABCA1 expression was approximately three fold higher compared to wild-type mice also treated with vehicle.

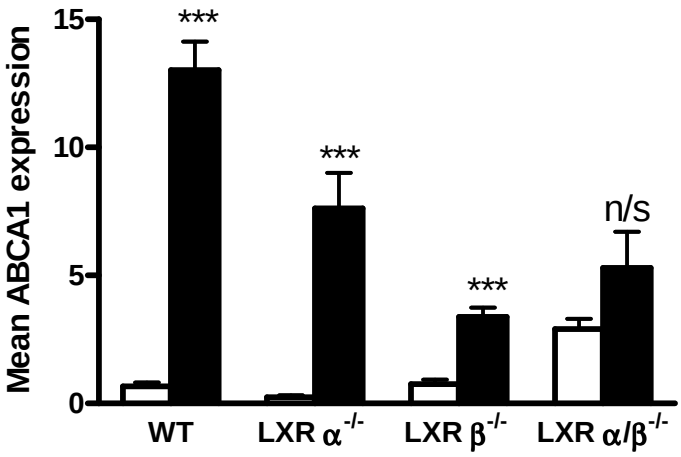


Figure 9.11 Induction of ABCA1 expression is diminished in LXR null mice.
Arthritis was induced in wild-type (*WT*), *LXRα*$^{-/-}$, *LXRβ*$^{-/-}$ or *LXRα/β KO*. Mice were treated daily with vehicle (5% mulgofen/PBS) (white bars) or 30 mg/Kg GW3965 (black bars) by IP injection. At cull jejunum was removed and the RNA extracted for gene expression analysis of ABCA1 by Taqman QRT-PCR. n = 9 per group, chosen at random which are representative of each genotype and treatment group. Students T test; *** P ≤ 0.001, not significant (n/s) (relative to vehicle group within the same genotype).

9.5 Discussion & conclusion

My previous studies suggested a novel pro-inflammatory effect of LXR agonism in the context of arthritis. However, these results were controversial to another study in the literature (125) and off target effects attributable to T1317 or GW3965 could not be discounted as potential drivers of inflammation and increased disease severity in CIA. Therefore, this study was initiated to determine if the pro-inflammatory effect observed upon administration of LXR agonists was mediated specifically by LXRs. Administration of GW3965 markedly increased the severity of arthritis in wild-type mice but not in *LXRα*$^{-/-}$, *LXRβ*$^{-/-}$ or *LXRα/β KO* suggesting a specific pro-inflammatory effect of dual LXRα/ LXRβ activation.

LXRα$^{-/-}$ and *LXRβ*$^{-/-}$ mice are on the C57BL/6 background which is generally considered resistant to CIA. However, Inglis *et al* recently published a study describing a detailed protocol for the induction of arthritis in C57BL/6 mice (225). Indeed, my preliminary experiments demonstrated that arthritis could be induced successfully in C57BL/6 following this protocol. However, it remains evident that C57BL/6 mice are generally more resistant to the development of arthritis than other strains of mice as disease is only observed in 50% of mice (Figure 9.1 and Figure 9.8), similar to previous reports (225, 301), in comparison to 80 to 100% incidence in DBA/1. Furthermore, the severity of disease is generally lower but more chronic in the C57BL/6, in comparison with DBA/1 mice, and is characterised with a sustained T cell response and anti-type II collagen antibodies (301). Consistent with these findings, DBA/1 mice treated with 30 mg/Kg GW3965 exhibited a mean arthritis score of 12 compared to 7 in C57BL/6 mice (compare Figure 8.26 to Figure 9.9). Although the ability to induce CIA in C57BL/6 mice is an extremely useful model the direct comparison of CIA, and the effect of LXR agonism, to DBA/1 is precluded.

To date the mechanism by which LXRs modulate inflammation is not fully understood and the individual role of LXRα or LXRβ in an inflammatory context is unknown. GW3965 and T1317 induce the activation of both LXR isoforms simultaneously and as isoform specific agonists have not been developed LXR KO mice are required to study the individual role of LXRα or LXRβ. Several studies

have also demonstrated that in mice deficient of either LXRα or LXRß the expression of some LXR target genes is maintained by the intact LXR isoform; for example the induction of ABCA1 expression is maintained in *LXRa-/-* mice (37, 101, 102). Therefore, to demonstrate that the pro-inflammatory effect observed with GW3965 administration was mediated specifically by LXRs it was necessary to generate *LXRa/ß KO* mice. Secondly, by inducing arthritis in *LXRa*$^{-/-}$ and *LXRß*$^{-/-}$ mice this will aid elucidation of the individual roles of the LXRs in inflammation. LXR deficient mice have been generated previously and whilst they are more susceptible to atherosclerosis i.e. increased LDL but reduced HDL cholesterol and accumulation of foam cells in arterial vessel walls, no other severe adverse phenotypes have been reported (299, 302). Indeed, this is the first study in which the individual role of LXRα and LXRß has been assessed in inflammation *in vivo*. Administration of 30 mg/Kg GW3965 significantly increased the severity of disease in wild-type but not LXR deficient mice. However, there was a large, but non-significant, increase in the paw swelling and clinical score in the *LXRa/ß KO* mice treated with GW3965 relative to vehicle. This is primarily caused by a high degree of variability and a lack of mice entered into these groups. Throughout these studies I have found that *LXRa/ß KO* mice, but not *LXRa*$^{-/-}$ or *LXRß*$^{-/-}$, are poor breeders and produce small litters generally between one to four pups. These observations have also been confirmed through personal communication with Prof David Mangelsdorf (Southwestern Medical Center, University of Texas). Due to the lack of mice the LXRα/ß KO group is under powered. However, disease severity is not altered in the *LXRa*$^{-/-}$ or *LXRß*$^{-/-}$ groups by treatment with GW3965. This suggests that the pro-inflammatory effect of GW3965 administration was mediated specifically by LXRs and not off target effects. Furthermore, these data suggest that cooperation between the two LXR isoforms or downstream effects is required to drive inflammatory pathways; at least in the context of arthritis.

LXRs have been primarily studied in the context of metabolism in which LXRα has been shown to primarily regulate the expression of genes involved lipogenesis (synthesis of fatty acids and triglycerides) whilst LXRß regulates the expression of genes involved in lipid transport and adipocyte gene expression (75, 112, 303). In particular LXRα regulates the expression of FAS and SREBP-1c in the liver and prolonged treatment of *LXRß*$^{-/-}$ mice with GW3965 or T1317 has

been shown to cause the development of hepatic steatosis; a potential driver of inflammation in arthritis (33, 81, 92). However, the severity of disease is not increased in *LXRβ*$^{-/-}$ mice that express LXRα. Although I have not measured lipid fatty acids and triglyceride concentrations these data suggest that hepatic steatosis is not the driver of inflammation and disease severity in arthritis. However, of interest the severity of arthritis in the vehicle treated groups is elevated in all the LXR deficient genotypes compared to wild-type mice (Figure 9.9 and Figure 9.10). The reason for this is unknown; however, as LXR null mice develop atherosclerosis it is clear that lipid pathways mediated by LXRs are impaired in LXR deficient mice. This may exert functional consequences upon inflammation in arthritis as several reports have demonstrated that deletion of the cholesterol transporter ABCA1, which is primarily regulated by LXRs, enhances the inflammatory response. Deletion of ABCA1 promotes macrophage polarisation to an M1 phenotype; increased STAT6 signalling, NF-κB activation and pro-inflammatory cytokine secretion, and neutrophil activation *in vivo* (304-307). Further in detail analysis of the innate and adaptive immune response including analysis of T cell phenotype, serum cytokines and antibodies is therefore required to determine the effect of LXR deletion upon inflammation in arthritis.

The expression of ABCA1 is regulated by both LXRα and LXRβ and is increased by approximately 13 fold in the jejunum of wild-type mice treated with GW3965 confirming activation of LXRs (96). Consistent with previous reports induction of ABCA1 expression was diminished in *LXRα*$^{-/-}$ mice but to a greater extent in *LXRβ*$^{-/-}$ (302). However, in *LXRα/β KO* mice although the expression of ABCA1 was not altered by GW3965 the basal level of ABCA1 expression was approximately three fold higher in LXRα/β KO mice compared to wild-type vehicle treated mice. The reason for this is unknown. However, inflammation inhibits reverse cholesterol transport *in vivo* in part mediated by the decreased expression of ABCA1 (308). Indeed my own data demonstrates that the expression of ABCA1 is decreased in murine LPS stimulated macrophages (Figure 10.7); similar results have also been demonstrated in murine kidney cells (41). Inhibition of ABCA1 expression has been suggested to be mediated in part through decreased LXRα expression and activation. Indeed, the recruitment of co-repressor complexes, e.g. NCoR, is a mechanism by which LXRs have been shown to repress the transcription of

several target genes including ABCA1, FAS and SREBP-1c (53). Therefore, inflammation may exert inhibitory effects mediated by LXRs upon the expression of LXR target genes which will be lost in LXRα/β KO mice potentially leading to elevated levels ABCA1 expression. However, further studies are required to confirm this hypothesis by examination of NCoR, and other co-repressors, recruitment to LXR heterodimers under an inflammatory context.

These data suggest that the increased severity of arthritis is mediated specifically through the activation of LXRα and LXRβ. However, the mechanism by which LXR agonism potentiates inflammation in the context of arthritis is unknown. Further studies are therefore required to elucidate the function of LXRα and LXRβ and the separate pathways they induce in an inflammatory context.

10 Analysis of Liver X Receptor activation in human & murine leukocytes

10.1 Aim & introduction

Whilst it is evident from other studies that activation of LXRs may exert anti-inflammatory effects my prior data clearly demonstrated that specific pharmacological activation of LXRs can promote inflammation *in vivo* in murine CIA. However, the potential role for LXRs in RA disease derived cells or tissues remains unknown.

The primary site of inflammation in RA is the synovium which contains high numbers of activated immune cells; macrophages, T cells, B cells and stromal derived fibroblasts in close proximity. Although the etiology of RA is unknown several mechanisms have been suggested by which inflammation in RA may be initiated and/or persist. Several studies have demonstrated increased expression of TLR3, TLR4 and TLR7/8 on RA derived synovial tissue leading to the hypothesis that viral and/or bacterial infection may promote synovitis (150, 152). Whilst various potential bacterial derived TLR ligands have been detected within the synovium more recently the presence of endogenous self ligands such as RNA and heat shock proteins released from necrotic synoviocytes have been shown to stimulate TLRs (149, 153). This observation not only provides support for a role for TLRs in mediating synovial inflammation but also suggests that the local environment within the synovium may potentiate the inflammatory response. Leukocyte activation may also occur through cellular interactions by a mechanism which is distinct from TLR ligation. For example, co-culture of synovial membrane derived T cells with syngeneic macrophages induces the secretion of macrophage derived TNFα in a cell contact dependent manner (162, 198). This can be recapitulated in an *in vitro* 'contact assay' by co-culture of cytokine-activated T cells (TcKs) with MCSF matured macrophages as an *in vitro* model of synovitis.

Although several studies have analysed the role of LXRs in inflammation these have been generally restricted to murine cells or rodent models of human disease. Both LXRα and LXRß have been shown to be expressed in a wide number of human immune cells including monocytes/ macrophages, T cells, dendritic cells, neutrophils and B cells (16-19, 118). However, how activation of LXRs affects inflammatory pathways in human cells, specifically in RA, and the

downstream impact upon human RA disease pathology remains unknown. *I therefore sought to determine how LXR agonism may impact upon inflammatory pathways in human cells that are relevant to RA disease pathology.*

10.2 LXR activation exerts differential species specific effects upon TLR4 stimulation

10.2.1 Optimisation of in vitro LXR agonist concentrations

The LXR agonists T1317 and GW3965 have been widely used as a tool to study the role of LXRs in physiology and disease. However, they have been utilised over a wide range of concentrations, especially *in vitro*, from as low as 0.01 μM up to 100 μM (33). To determine an optimal concentration range of T1317 which sufficiently induces the expression of LXR target genes, monocytes were purified from the peripheral blood of healthy volunteers and treated with vehicle (DMSO) or T1317 for twenty four hours. A range of 2 μM to 20 μM T1317 was selected from initial pharmacological studies which identified T1317 as an LXR agonist in mice (33). The RNA was extracted and analysed by Taqman QRT-PCR from which the fold increase of ABCA1 expression was used as a reporter of LXR activation (Figure 10.1). T1317 induced the expression of ABCA1 at all concentrations by approximately 8 fold at 2 μM T1317 to 12 fold with 20 μM T1317 relative to media alone. However, there was no difference in the expression of ABCA1 above 6 μM T1317 suggesting maximal stimulation had been achieved. Therefore, to identify a sub-optimal concentration range of T1317 which induced the expression of ABCA1 in a dose-responsive manner, primary human monocytes were treated with vehicle or 0.01 μM to 4 μM T1317. The expression of ABCA1 was measured by Taqman QRT-PCR to determine the level of LXR transcriptional activation (Figure 10.2). T1317 induced the expression of ABCA1 in a dose responsive manner by approximately 10 to 40 fold with 0.01 μM and 4 μM T1317 respectively. T1317 has been shown to non-specifically activate PXR and FXR and as the expression of the LXR specific reporter gene ABCA1 was not different with concentrations of T1317 above 4 μM, concentrations of T1317 between 0.01 μM and 4 μM were selected for future human *in vitro* analysis (297, 298).

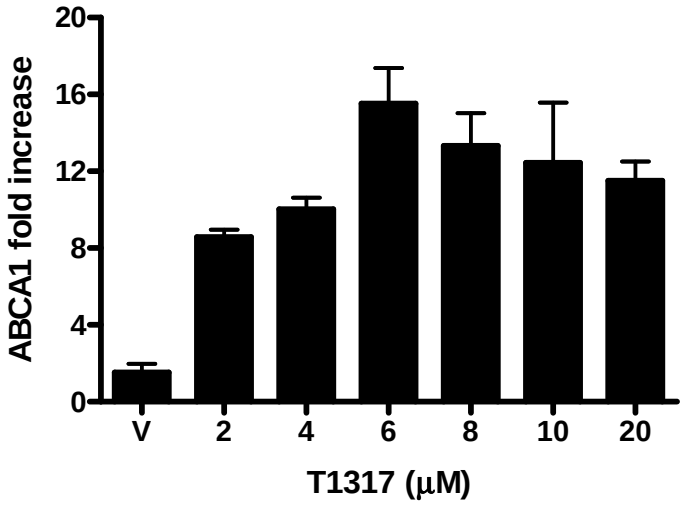


Figure 10.1 Induction of LXR activation by T1317 in primary human monocytes.
Primary human monocytes were cultured with media alone, vehicle (V - DMSO) or T1317 at the indicated concentrations for 24 hours. The fold increase of ABCA1 expression relative to cells treated with media alone was measured by Taqman QRT-PCR and normalised to TATA binding protein (TBP). Each condition was tested in triplicate.

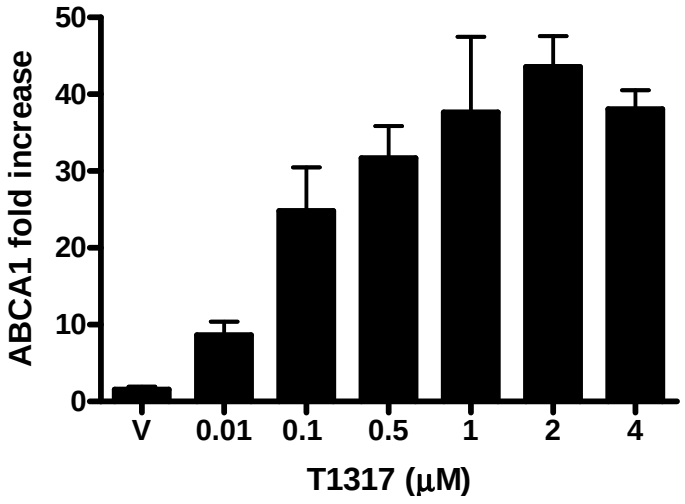


Figure 10.2 The expression of ABCA1 is induced with by T1317 with concentrations between 0.01 to 4 μM.
Primary human monocytes were cultured with media alone, vehicle (V - DMSO) or T1317 at the indicated concentrations for 24 hours. The fold increase of ABCA1 expression relative to cells treated with media alone was measured by Taqman QRT-PCR normalised to TAT binding protein (TBP). Each condition was tested in triplicate.

10.2.2 LXR activation inhibits IL-6 secretion from TLR stimulated murine macrophages.

A regulatory role for LXR activation upon pro-inflammatory cytokine secretion was first demonstrated by Joseph *et al* in which LXR agonism suppressed the expression of iNOS in murine macrophages stimulated with LPS or infected with E.coli (117). To confirm this anti-inflammatory effect of LXR agonism, bone

marrow derived macrophages (BMDM) from male wild-type C57BL/6 mice were treated with 1 μM to 4 μM GW3965, concentrations similar to that used by Joseph *et al*, for 24 hours and then stimulated with 100 ng/ml LPS. After twenty four hours, the concentration of the secreted pro-inflammatory cytokine IL-6 was measured by ELISA (Figure 10.3). Treatment of LPS stimulated macrophages with GW3965 significantly inhibited the secretion of the pro-inflammatory cytokine IL-6. These data were consistent with prior observations and confirmed that my methodological approach was sufficiently sound to address the relative contribution of LXRs towards cytokine secretion.

More recent reports in human monocytes suggested that LXR agonists could exert differential effects upon the secretion of pro-inflammatory cytokines dependent upon the length of time that cells were pre-incubated with LXR agonists (61). Therefore, to determine if different pre-incubation times might effect the secretion of IL-6 murine BMDMs were pre-incubated with 4 μM GW3965 for 6 hrs, 9 hrs, 24 hrs and 48 hrs; time points which are comparable to the previous report, prior to stimulation with 100 ng/ml LPS (Figure 10.4). The concentration of IL-6 secreted into the cell culture supernatants was significantly decreased at all time points but to a greater extent with longer periods of GW3965 pre-incubation.

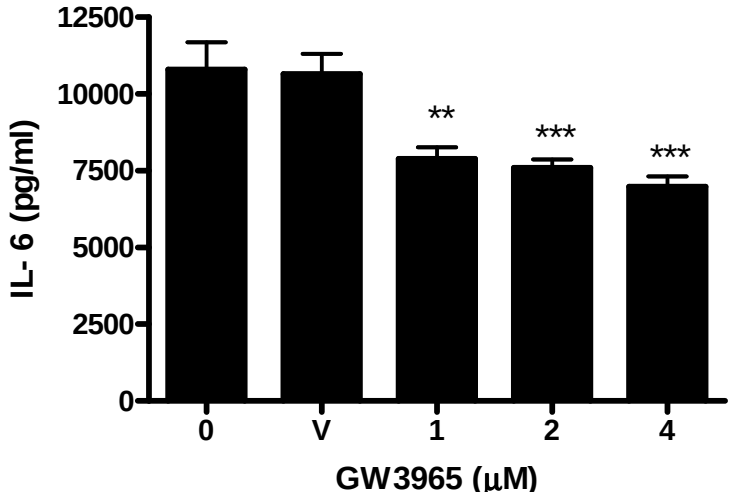


Figure 10.3 LXR agonism inhibits the secretion of IL-6 from LPS stimulated murine macrophages.
Bone marrow derived macrophages were treated with media alone (0), vehicle (V – DMSO) or GW3965 at the indicated concentrations for 24 hours after which they were stimulated with 100 ng/ml LPS. The concentration of IL-6 was measured by ELISA. Each condition was tested in triplicate with macrophages derived from 5 individual mice. Students T test; ** P ≤ 0.01, *** P ≤ 0.001.

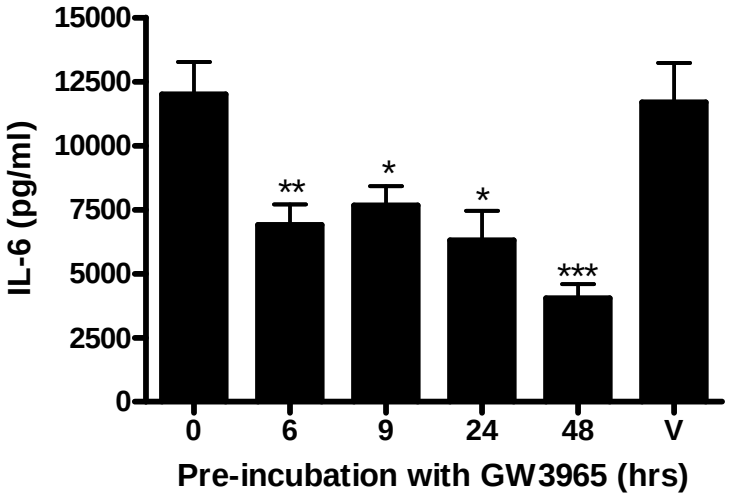


Figure 10.4 Prolonged pre-incubation with GW3965 further inhibits the secretion of IL-6. Bone marrow derived macrophages were treated with 4 μM GW3965 for the indicated number of hours or vehicle (V –DMSO) for 48 hours prior to stimulation with 100 ng/ml LPS. The concentration of IL-6 was measured by ELISA. Each condition was tested in triplicate with macrophages derived from 5 individual mice. Students T test; * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001.

My results are consistent with the studies of Joseph *et al* (117) which suggested an anti-inflammatory effect of LXR activation in the context of bacterial infection and TLR4 ligation in murine macrophages. However, the effect of LXR agonism upon pro-inflammatory cytokine secretion mediated by the ligation of other TLRs was unknown. BMDMs were pre-incubated for 48 hrs with 4 μM GW3965 prior to stimulation with various TLR ligands; 100 ng/ml LPS (TLR4), 1 μg/ml PAM3Cys (TLR2), 1 μg/ml Lipoteichoic acid (LTA - TLR2), 10 μg/ml Poly IC (TLR3), 1 μg/ ml CL97 (TLR7/8) and 1 μM CpG (TLR9). After stimulation for 24 hours, the concentration of IL-6 in cell culture supernatants was measured by ELISA (Figure 10.5). GW3965 significantly decreased the secretion of IL-6 in cells stimulated with LPS, LTA and PAM relative to vehicle. PiC, CpG and CL97 failed to induce the secretion of IL-6. This study demonstrates that LXR agonism is able to suppress cytokine secretion mediated by other TLR ligands in murine macrophages.

To demonstrate that inhibition of IL-6 secretion induced by PAM and LTA as well as LPS was mediated specifically by LXRs, BMDMs were differentiated from male wild-type, *LXRα*$^{-/-}$ and *LXRβ*$^{-/-}$ mice on the C57BL/6 background. The cells were pre-incubated for 48 hrs with 4 μM GW3965 prior to stimulation with TLR ligands at the indicated concentrations (Figure 10.6). Consistent with my previous

results treatment of LPS, PAM or LTA stimulated BMDMs from wild-type mice with 4 μM GW3965 significantly inhibited the secretion of IL-6. Supporting an anti-inflammatory effect of LXRs, deletion of LXRα or LXRß was associated with an increased concentration of IL-6 upon ligation of TLR4. Furthermore, the inhibitory effect of GW3965 upon the secretion of IL-6 in response to TLR 4 ligation was diminished in BMDMs from *LXRα*$^{-/-}$ and *LXRß*$^{-/-}$mice. These data suggest that the anti-inflammatory effect observed after treatment of BMDMs with GW3965 is mediated specifically through the activation of LXRs. Interestingly, inhibition of IL-6 secretion by treatment with GW3965 in response to stimulation with PAM or LTA appears to be mediated specifically through the actions of LXRß. This is evident as deletion of LXRß, but not LXRα, is associated with increased concentrations of IL-6 and GW3965 was not effective at reducing the concentrations of IL-6 in the LXRß$^{-/-}$ macrophages.

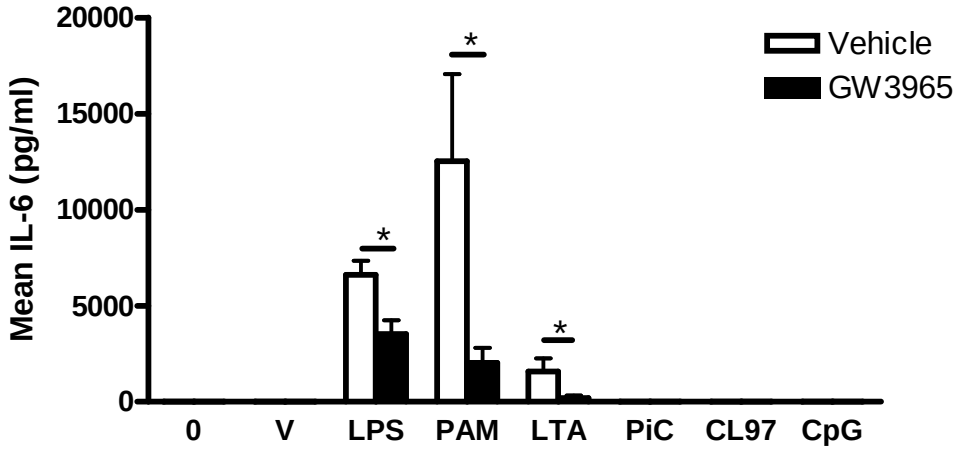


Figure 10.5 LXR activation inhibits IL-6 secretion from TLR activated murine macrophages. Bone marrow derived macrophages were pre-incubated for 48 hrs with vehicle (V – DMSO) or 4 µM GW3965 prior to stimulation with the following TLR ligands; 100 ng/ml LPS (TLR4), 1 µg/ml PAM3Cys (TLR2), 1 µg/ml Lipoteichoic acid (LTA - TLR2), 10 µg /ml Poly IC (PiC - TLR3), 1 µg/ ml CL97 (TLR7/8) and 1 µM CpG (TLR9). The concentration of IL-6 was measured by ELISA. Students T test; * P ≤ 0.05. n = 4 per group.

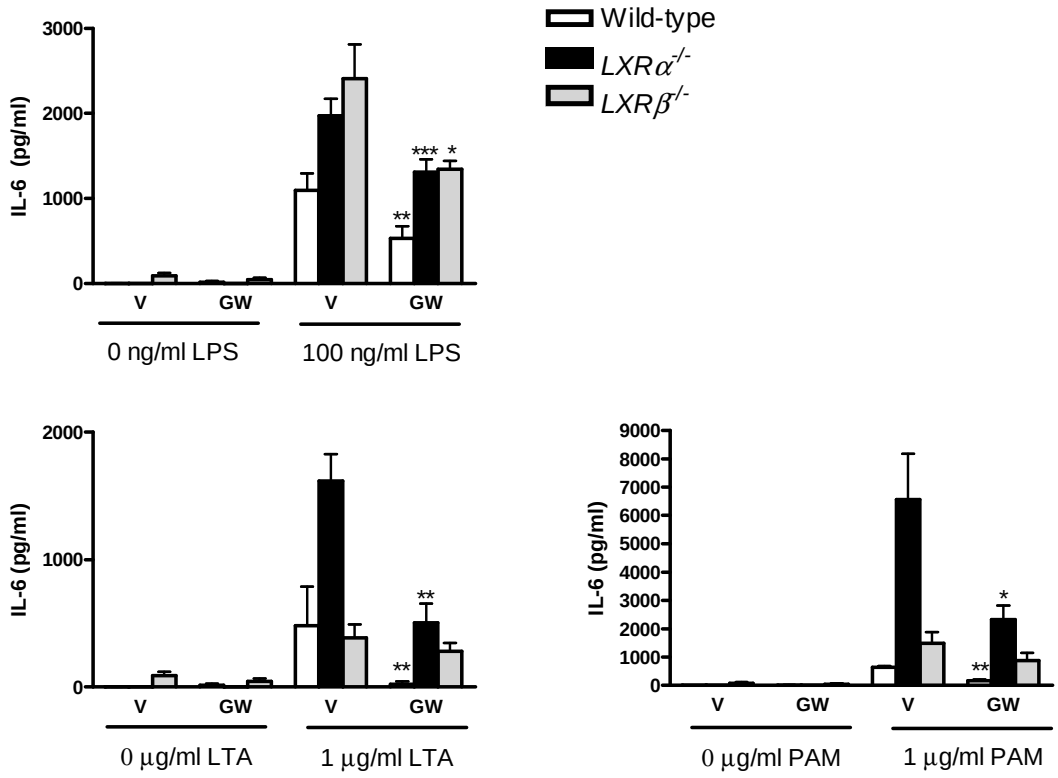


Figure 10.6 Inhibition of TLR induced IL-6 secretion is mediated specifically by LXRs. Bone marrow derived macrophages were differentiated from male wild-type, *LXRα*$^{-/-}$ and *LXRβ*$^{-/-}$ mice and pre-incubated for 48 hrs with vehicle (V – DMSO) or 4 µM GW3965 (GW) prior to stimulation with the following TLR ligands; 100 ng/ml LPS (TLR4), 1 µg/ml PAM3Cys (PAM -TLR2), 1 µg/ml Lipoteichoic acid (LTA - TLR2). The mean concentration of IL-6 was measured by ELISA. Students T test; *** P ≤ 0.001, ** P ≤ 0.01 relative to vehicle control. n = 4/ group.

10.2.3 LXR activation synergises with TLR4 ligation to promote pro-inflammatory cytokine secretion

The role of LXRs, especially in an inflammatory context, has been predominantly studied in mice or murine cells in which it is evident that LXR agonism can exert an anti-inflammatory effect upon TLR ligation in murine macrophages. The effect of LXR agonism upon TLR ligation in human macrophages was unknown. To evaluate the effect of LXR activation upon cytokine secretion in humans $CD14^+$ monocytes were purified from the peripheral blood of healthy volunteers. The monocytes were pre-incubated for twenty four hours with vehicle (DMSO) or T1317 prior to stimulation with 100 ng/ml LPS. Taqman QRT-PCR for the increased expression of ABCA1 confirmed activation of LXRs by T1317 that was significantly inhibited by the addition of LPS (Figure 10.7). In contrast to the effect of LXR activation in murine macrophages, treatment of LPS stimulated human monocytes with T1317 increased the secretion of the pro-inflammatory cytokine IL-6 in a dose responsive manner (Figure 10.8). Treatment of monocytes with T1317 alone did not induce IL-6 secretion thus indicating that there was no contamination with any TLR ligands that could potentiate the inflammatory response.

To determine if the pro-inflammatory effect of LXR agonism was specific to IL-6 the concentration of TNFα in the same cell culture supernatants was measured by ELISA (Figure 10.9). In accordance with the increased concentrations of IL-6, LXR agonism by T1317 also increased the concentration of TNFα from LPS stimulated monocytes. These data suggest that LXR agonism exerts a general pro-inflammatory effect. Therefore, to determine the cytokine profile induced by LXR activation and TLR4 ligation, Luminex analysis was used to measure the concentration of multiple pro-inflammatory cytokines and chemokines (Figure 10.10 and Figure 10.11). In support of a pro-inflammatory effect of LXR agonism the concentration of several pro-inflammatory cytokines, namely IL-1ß, IL-6, IL-7, IL-12 and IL-17, and the inflammatory chemokines, MIP-1α, MIP-1ß and RANTES, were increased in cell culture supernatants whilst the concentration of the anti-inflammatory cytokine IL-10 was significantly reduced. IL-2, IL-4, IL-5 and IFNγ were not detectable and IL-8 was above the limits of assay detection. In agreement with my results, other studies have since demonstrated a pro-

inflammatory effect of LXR agonists in LPS stimulated human macrophages in which they demonstrated that the elevated levels of MCP-1 in the presence of LXR agonists was mediated through the increased expression of TLR4 (61). I have since confirmed the findings of this study and have also demonstrated that the expression of TLR4 in human monocytes was increased by LXR activation (Figure 10.12).

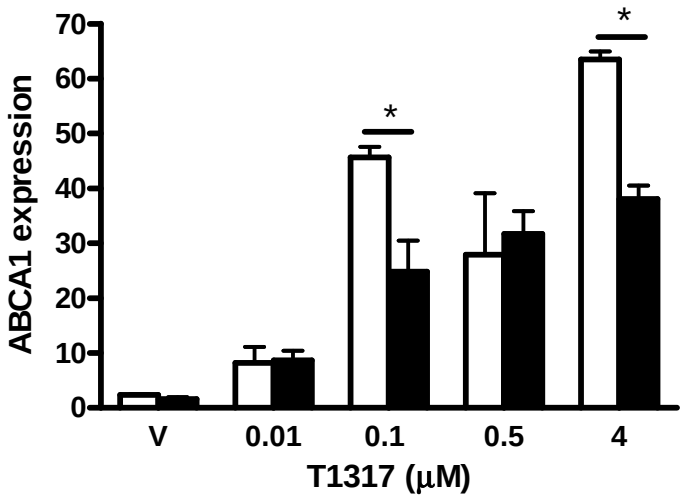


Figure 10.7 TLR4 ligation inhibits LXR induced transcription in primary human monocytes. Human CD14^{+} monocytes were pre-incubated for 24 hrs with media alone (0), vehicle (V – DMSO) or T1317 prior to stimulation with 100 ng/ml LPS (black bars) or media (white bars). The fold increase of ABCA1 expression relative to media alone was measured by Taqman QRT-PCR and normalised to TATA binding protein (TBP). Each condition was tested in triplicate. Students T test; * $\leq$ 0.05.

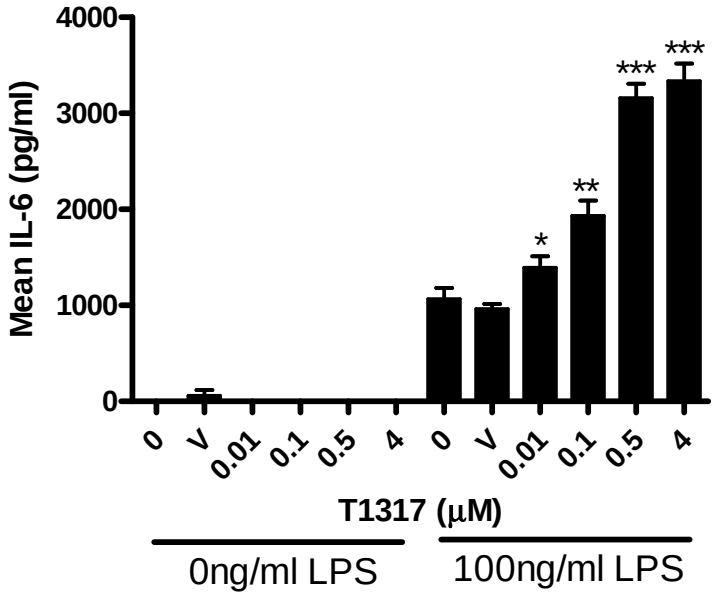


Figure 10.8 LXR activation increases the secretion of IL-6 from human LPS stimulated monocytes.
Human CD14$^+$ primary monocytes were pre-incubated for 24 hrs with media alone (0), vehicle (V – DMSO) or T1317 at the indicated concentrations prior to stimulation with 100 ng/ml LPS. The concentration of IL-6 in cell culture supernatants was measured by ELISA. Each condition was tested in triplicate. These data are representative of results from 6 individual donors. Students T test; * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001.

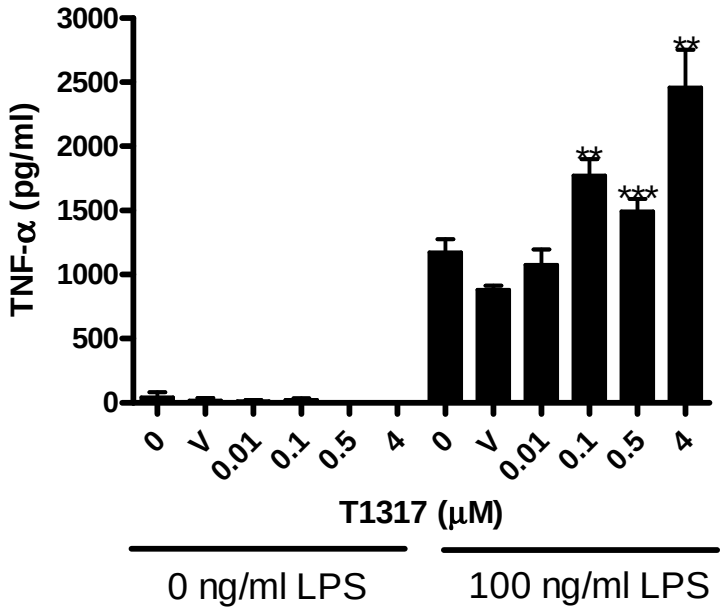


Figure 10.9 LXR agonism increases the secretion of TNFα from LPS stimulated human monocytes.
Human CD14$^+$ monocytes were pre-incubated for 24 hrs with media alone (0), vehicle (V – DMSO) or T1317 at the indicated concentrations prior to stimulation with 100 ng/ml LPS. The concentration of TNFα in cell culture supernatants was measured by ELISA. Each condition was tested in triplicate. Students T test; ** P ≤ 0.01, *** P ≤ 0.001.

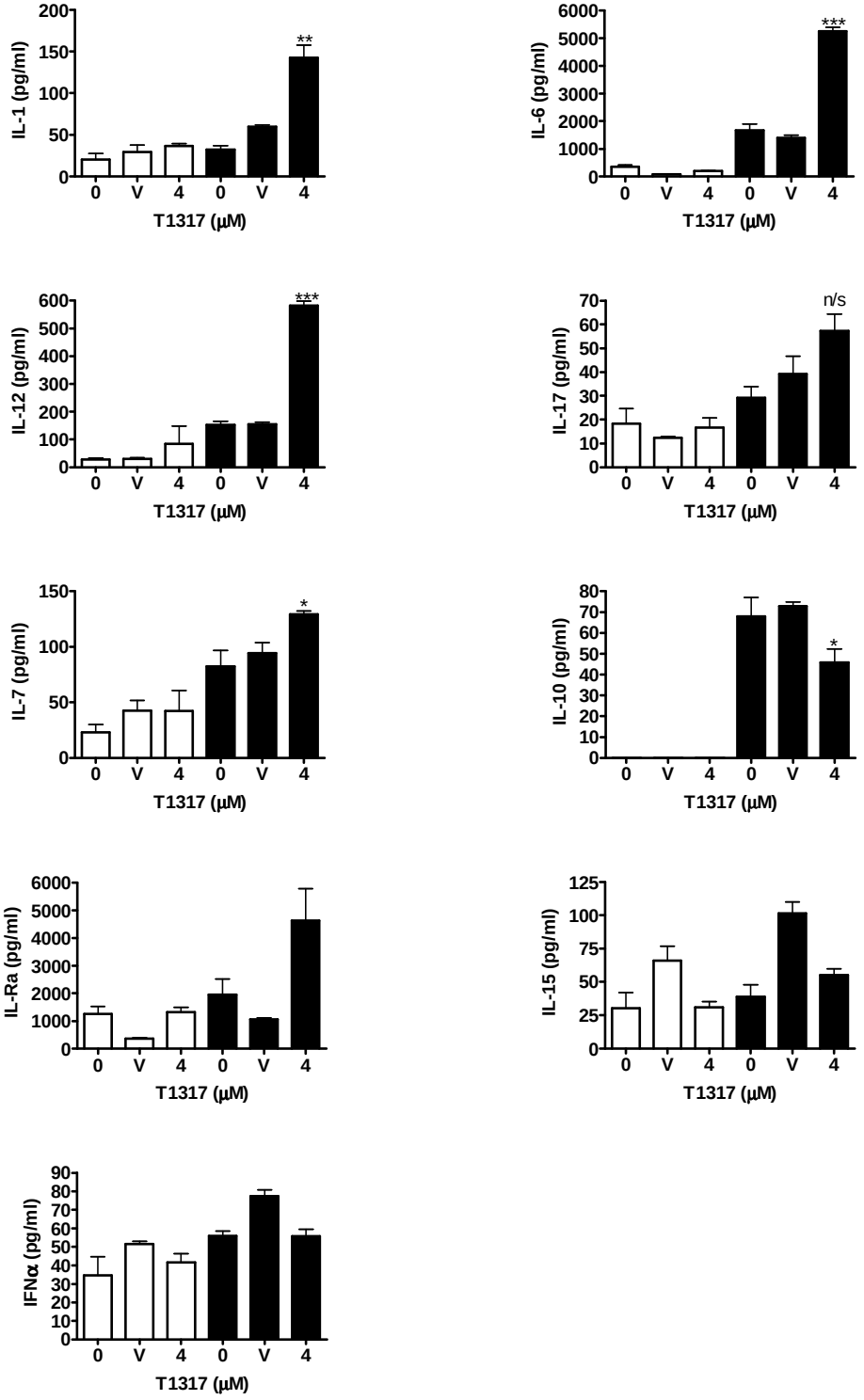


Figure 10.10 LXR agonism increases the secretion of multiple pro-inflammatory cytokines from LPS stimulated human monocytes.
Human CD14^{+} monocytes were pre-incubated for 24 hrs with media alone (0), vehicle (V – DMSO) or 4 µMT1317 prior to stimulation with media alone (white bars) or 100 ng/ml LPS (black bars). The concentration of cytokines in cell culture supernatants was measured by Luminex. Each condition was tested in triplicate. Students T test; * $\leq$ 0.05, ** $\leq$ 0.01 * $\leq$ 0.001.**

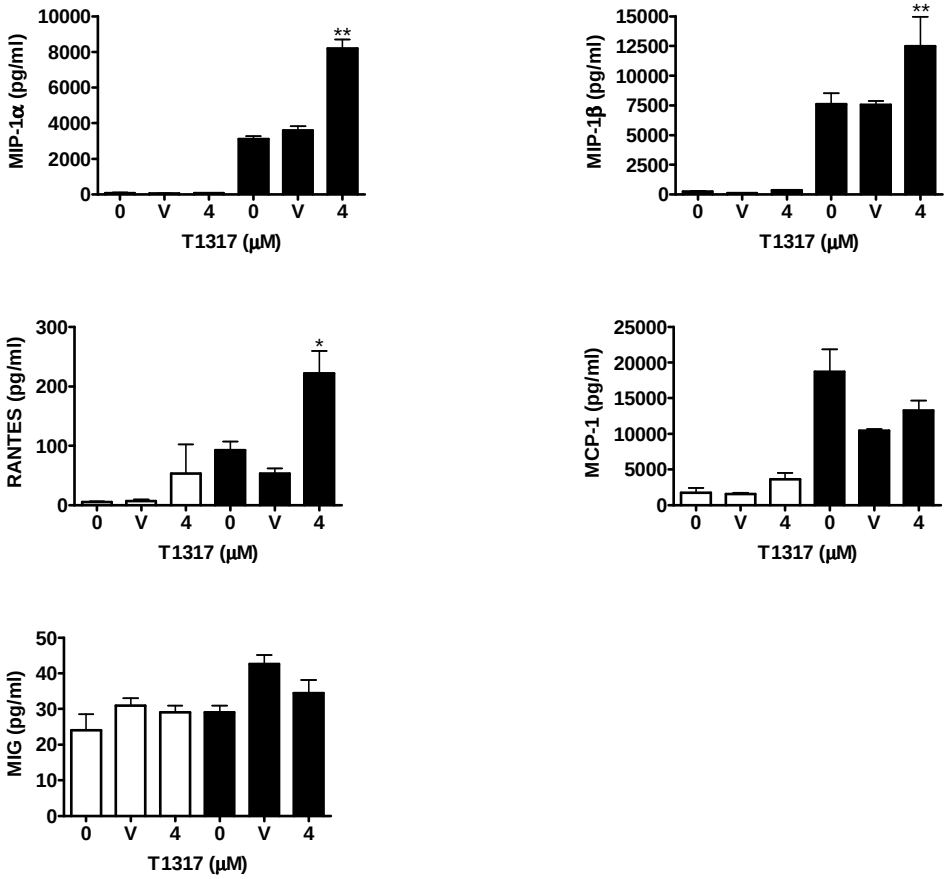


Figure 10.11 The secretion of inflammatory chemokines is increased by LXR activation. Human CD14^{+} monocytes were pre-incubated for 24 hrs with media alone (0), vehicle (V – DMSO) or 4 μMT1317 prior to stimulation with 100 ng/ml LPS. The concentration of inflammatory chemokines in cell culture supernatants was measured by Luminex. Each condition was tested in triplicate. Students T test; * ≤ 0.05, ** ≤ 0.01.

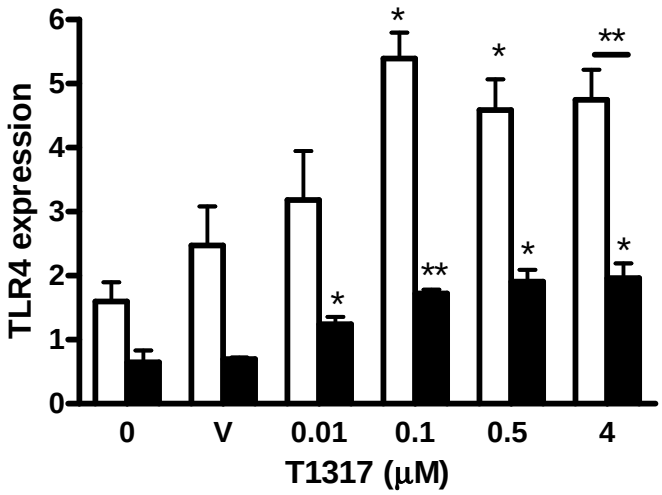


Figure 10.12 LXR activation increased the expression of TLR4.
Human CD14$^+$ monocytes were pre-incubated for 24 hrs with media alone (0), vehicle (V – DMSO) or T1317 prior to stimulation with 100 ng/ml LPS (black bars) or media (white bars). The expression of TLR4 normalised to TATA binding protein (TBP) was measured by Taqman QRT-PCR. Each condition was tested in triplicate. Students T test; * $\leq$ 0.05 (relative to vehicle), ** P $\leq$ 0.01.

The importance of an LXR driven pathway in the pathology of RA is unknown. Therefore, to determine if the pro-inflammatory effect of LXR activation upon TLR4 ligation is conserved in patients with RA, CD14$^+$ monocytes were purified from RA peripheral blood. As previously described, the cells were pre-treated with media, vehicle, T1317 or GW3965 for 24 hours prior to stimulation with 100 ng/ml LPS. LXR agonism significantly increased the concentration of LPS induced secretion of IL-6 and TNFα in cell culture supernatants as measured by ELISA in a dose responsive manner (Figure 10.13). Of interest, in the absence of LPS although both TNFα and IL-6 were secreted at low concentrations, reflecting the systemic pro-inflammatory environment in RA; LXR agonism did not alter the level of cytokine secretion. These results demonstrate that LXR agonism is able to induce pro-inflammatory effects in LPS stimulated monocytes in samples from both healthy controls and patients with RA.

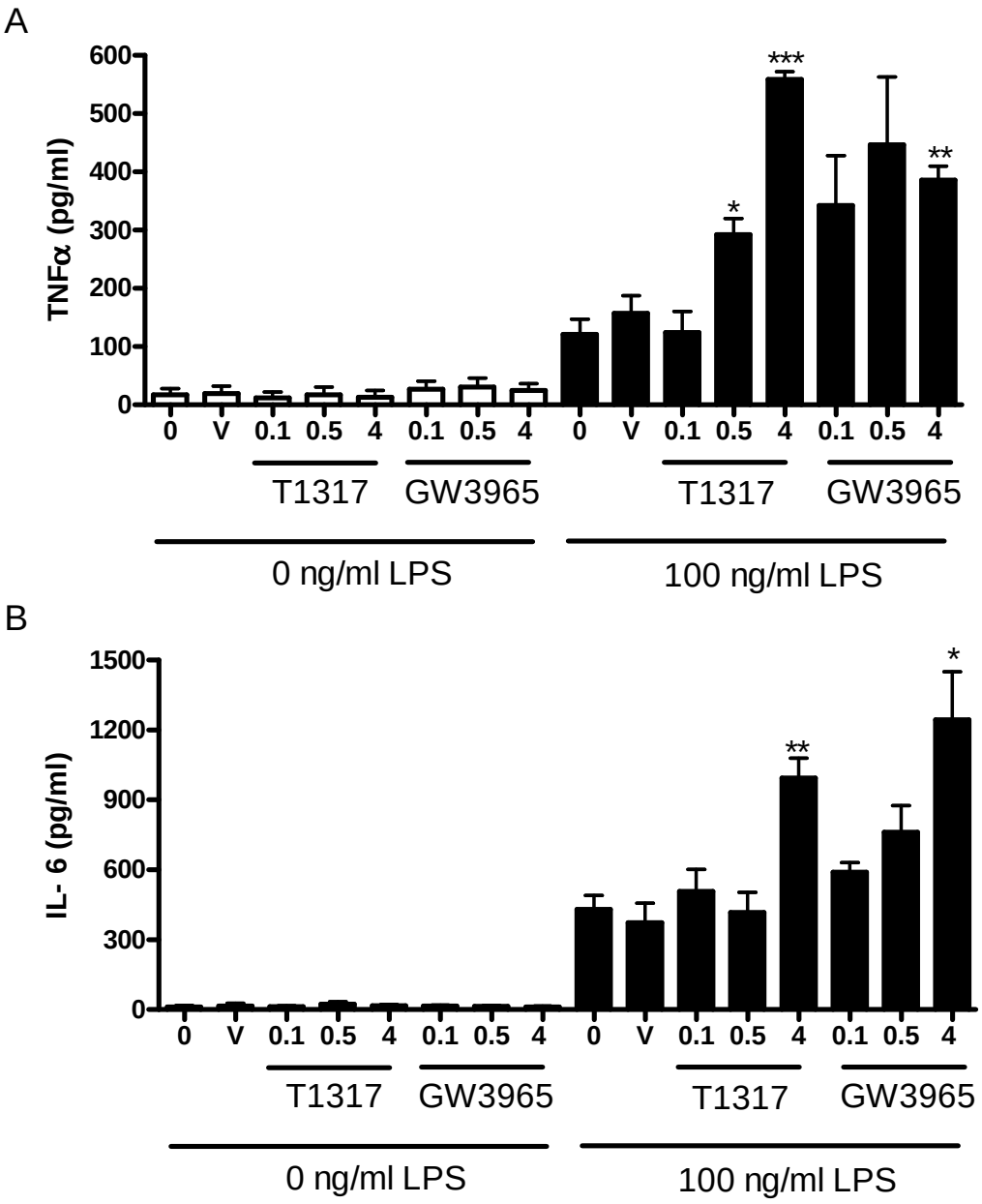


Figure 10.13 LXR agonism promotes cytokine secretion from LPS stimulated RA monocytes.
(A – B) Monocytes were purified from the peripheral blood of patients with RA and incubated for 24 hrs with media alone (0), vehicle (V – DMSO), T1317 and GW3965 at the indicated concentrations prior to stimulation with 100 ng/ml LPS. The concentration of TNFα (A) and IL-6 (B) was measured by ELISA. Each condition was tested in triplicate and the results are representative of 3 independent donors. Students T test; * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001.

10.2.4 LXR activation has no effect upon pro-inflammatory cytokine secretion from inflamed synovial membrane

The effect of LXR activation at the primary site of inflammation within the RA synovium has not previously been defined. Both LXRα and LXRß are expressed

within cells derived from collagenase digested RA synovial membrane (synoviocytes) and synovial derived fibroblasts from patients with RA (Figure 10.14). Therefore, to determine if LXR activation could potentially increase the basal level of cytokine secretion within the synovium freshly isolated RA derived synoviocytes were treated with vehicle or T1317 (Figure 10.15). In the absence of any exogenous inflammatory stimuli, the RA derived synoviocytes spontaneously secreted high concentrations of TNFα. However, addition of T1317 or GW3965 did not affect the secretion of TNFα. Fibroblasts are the major cell type of the synovial membrane. Therefore, fibroblasts derived from inflamed RA synovial membrane were treated with T1317 and stimulated with 100 ng/ml LPS (Figure 10.16). However, despite induction of the TLR4 pathway, evident by the increased secretion of IL-6, LXR agonism did not further increase the concentration of IL-6 secreted from RA fibroblasts. Similar results were also obtained in RA synoviocytes (Figure 10.17).

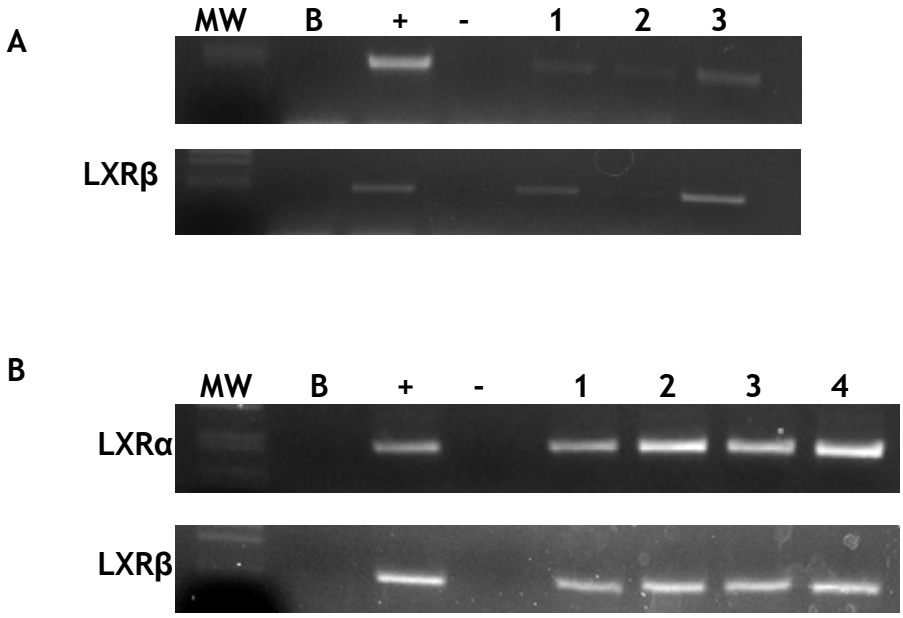


Figure 10.14 LXRs are expressed in synoviocytes and synovial fibroblasts.
(A – B)The expression of LXRα and LXRβ was assessed by RT-PCR. (A) RNA was extracted from synovial membrane from RA patients following collagenase digestion; n =3 (A) and RA synovial derived fibroblast explant cultures at passage 5; n = 4 (B). Molecular weight ladder (MW), blank (B – no RNA) positive control (+, human monocytes), negative control (- , without reverse transcriptase).

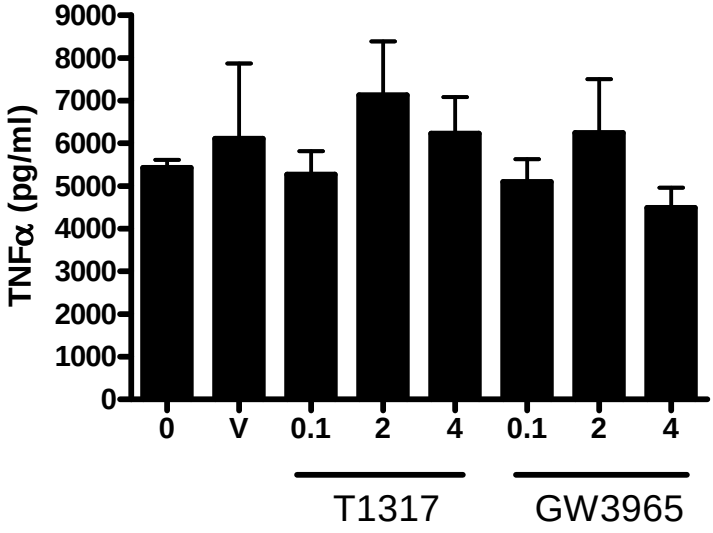


Figure 10.15 LXR agonism does not promote cytokine secretion from synoviocytes.
Synoviocytes derived from rheumatoid arthritis synovial membrane following collagenase digestion were treated with media alone (0), vehicle (V – DMSO), T1317 or GW3965 at the indicated concentration for 24 hrs. The concentration of TNFα was measured by ELISA. Each condition was tested in triplicate. The results are representative 3 separate donors.

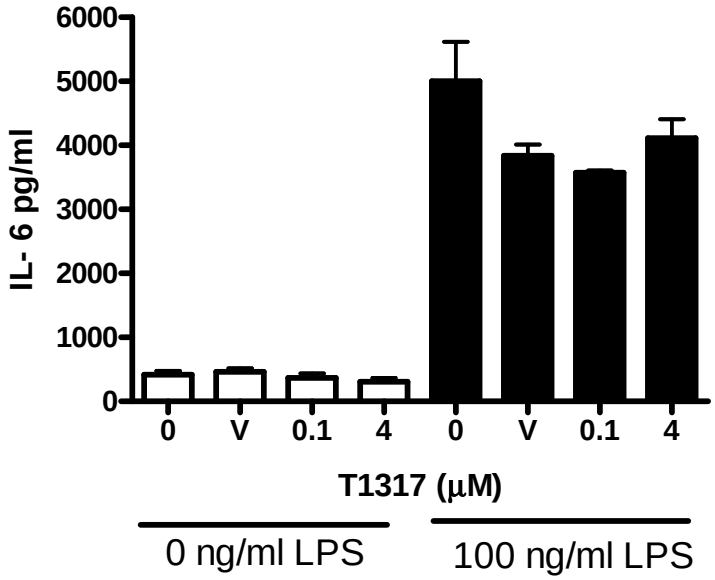


Figure 10.16 LXR activation does not increase secretion of IL-6 from LPS stimulated rheumatoid synovial fibroblasts.
RA synovial fibroblast explant cultures at passage 5 were pre-incubated for 24 hrs with media alone (0), vehicle (V – DMSO) or T1317 at the indicated concentrations prior to stimulation with 100 ng/ml LPS. The concentration of IL-6 was measured by ELISA. Each condition was tested in triplicate. The results are representative 5 separate donors.

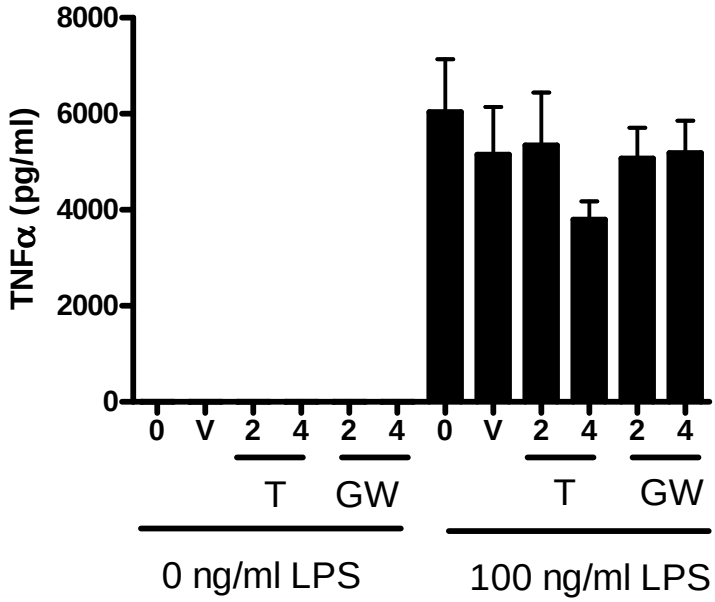


Figure 10.17 LXR agonism does not promote TNFα secretion from LPS stimulated synoviocytes.
Synoviocytes derived from RA synovial membrane following collagenase digestion were treated with media alone (0), vehicle (V – DMSO), T1317 (T) or GW3965 (GW) at the indicated concentration for 24 hrs prior to stimulation with 100 ng/ml LPS. The concentration of TNFα was measured by ELISA. Each condition was tested in triplicate. The results are representative of 3 separate donors.

10.2.5 LXRs exacerbate cytokine secretion in an in vitro model of synovitis

LXR agonism supports the secretion of inflammatory cytokines and chemokines from human LPS stimulated monocytes but not RA synovial tissue derived synoviocytes or fibroblasts. The effect of LXR agonism upon other inflammatory pathways relevant to RA remains unknown. Whilst RA is considered a Th1 disease synovial T cells have a phenotype more similar to cytokine activated T cells (TcKs) which are able to induce macrophage activation and consequently the secretion of macrophage derived pro-inflammatory cytokines including TNFα (162). To determine if LXR activation has the potential to drive an inflammatory response through the interaction of macrophages and TcKs, monocytes were purified from the peripheral blood of healthy controls and matured to a macrophage phenotype with MCSF in the presence of vehicle, GW3965 or T1317. Syngeneic $CD3^+$ T cells were simultaneously purified and activated with IL-2, IL-6 and TNFα. After seven days, macrophages and TcKs were co-cultured for 24 hours and the concentration of macrophage derived TNFα was measured by ELISA (Figure 10.18). Co-culture of macrophages and TcKs induced the secretion of TNFα which was significantly increased by treatment with T1317 or GW3965 in a dose responsive manner. Luminex analysis was used to assess the effect of LXR agonism upon the secretion of other inflammatory cytokines and chemokines (Figure 10.19 and Figure 10.20). Indeed, the concentration of multiple pro-inflammatory cytokines IL-1ß, IL-2, IL-5, IL-6, IL-12, IL-13, IL-15, IL-17, GM-CSF, IFNγ and TNFα, were significantly increased in cell culture supernatants by T1317 and GW3965. Similarly, both T1317 and GW3965 significantly increased the concentration of the inflammatory chemokines MIP-1α and MIP-1ß. The concentration of IL-1Ra, IFNα, IL-4, IL-7, RANTES, IP-10, MIG and MCP-1 were not changed, whilst IL-8 and eotaxin were outwith the limits of assay detection. These data suggest that LXR agonism may be able to promote an inflammatory response mediated through the interaction of cytokine activated T cells and macrophages within an inflamed RA synovium.

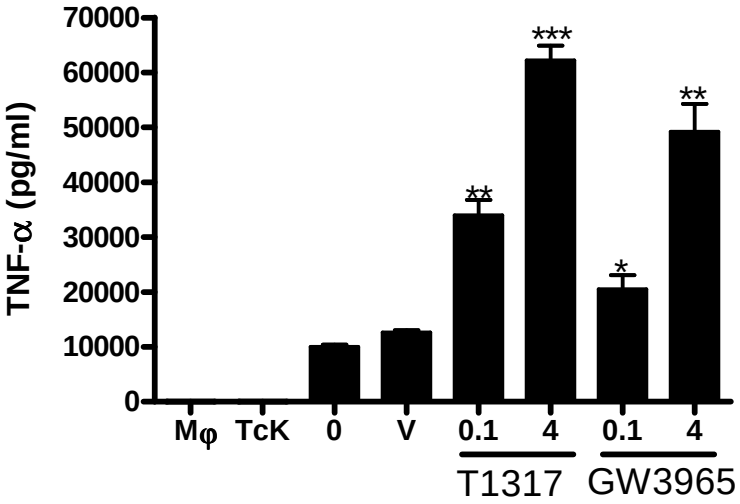
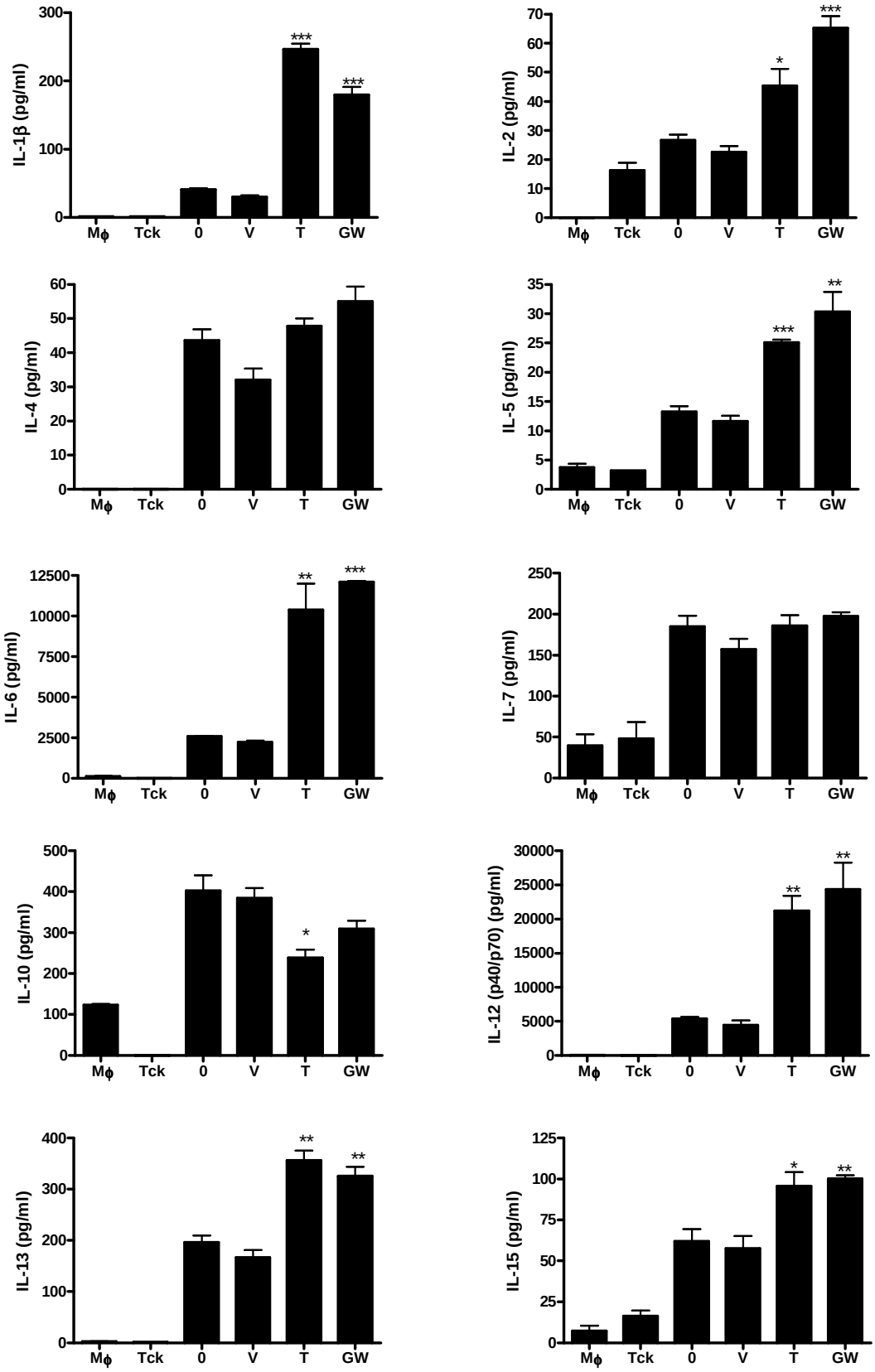


Figure 10.18 LXR agonism increases the secretion of TNFα in an *in vitro* model of synovitis. Primary human monocytes were purified and matured to a macrophages (MΦ) phenotype with MCSF. Simultaneously, unfixed syngeneic $CD3^+$ T cells were activated with IL-2, IL-6 and TNFα (cytokine activated T cells- TcKs). After seven days the cells were co-cultured and incubated overnight in the presence of media alone (0), vehicle (V – DMSO), T1317 or GW3965 at the indicated concentration. The concentration of TNFα was measured by ELISA. Each condition was tested in triplicate and the results are representative of 6 different donors. Students T test; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.



LXR agonism promotes inflammatory cytokine secretion from T cell activated macrophages, (continued over).

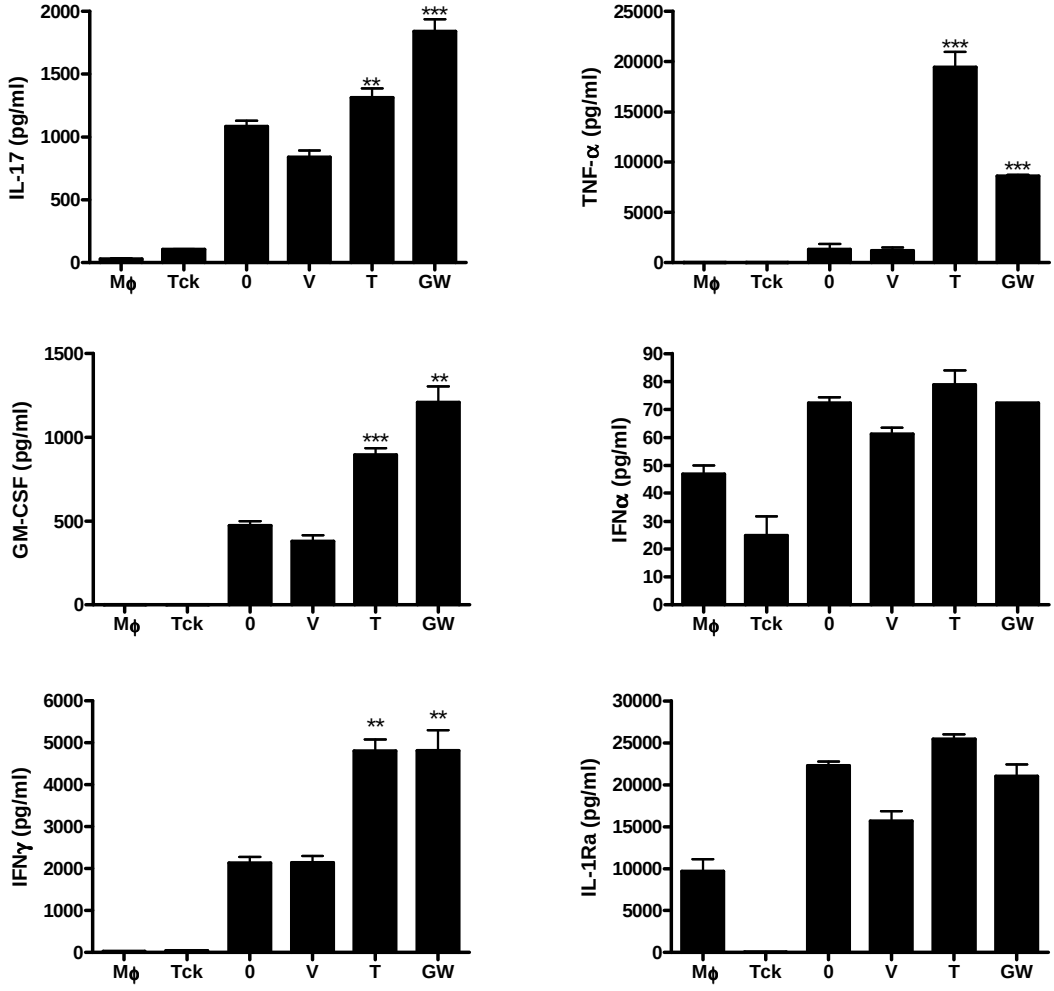


Figure 10.19 LXR agonism promotes inflammatory cytokine secretion from T cell activated macrophages.
Primary human monocytes were purified and matured to a macrophages (MΦ) phenotype with MCSF. Simultaneously, syngeneic $CD3^+$ T cells were activated with IL-2, IL-6 and TNFα (cytokine activated T cells- TcKs). After seven days the cells were co-cultured and incubated overnight in the presence of media alone (0), vehicle (V – DMSO), 4 μM T1317 (T) or 4 μM GW3965 (GW). The concentration of cytokines was measured by Luminex. Each condition was tested in triplicate. Students T test; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

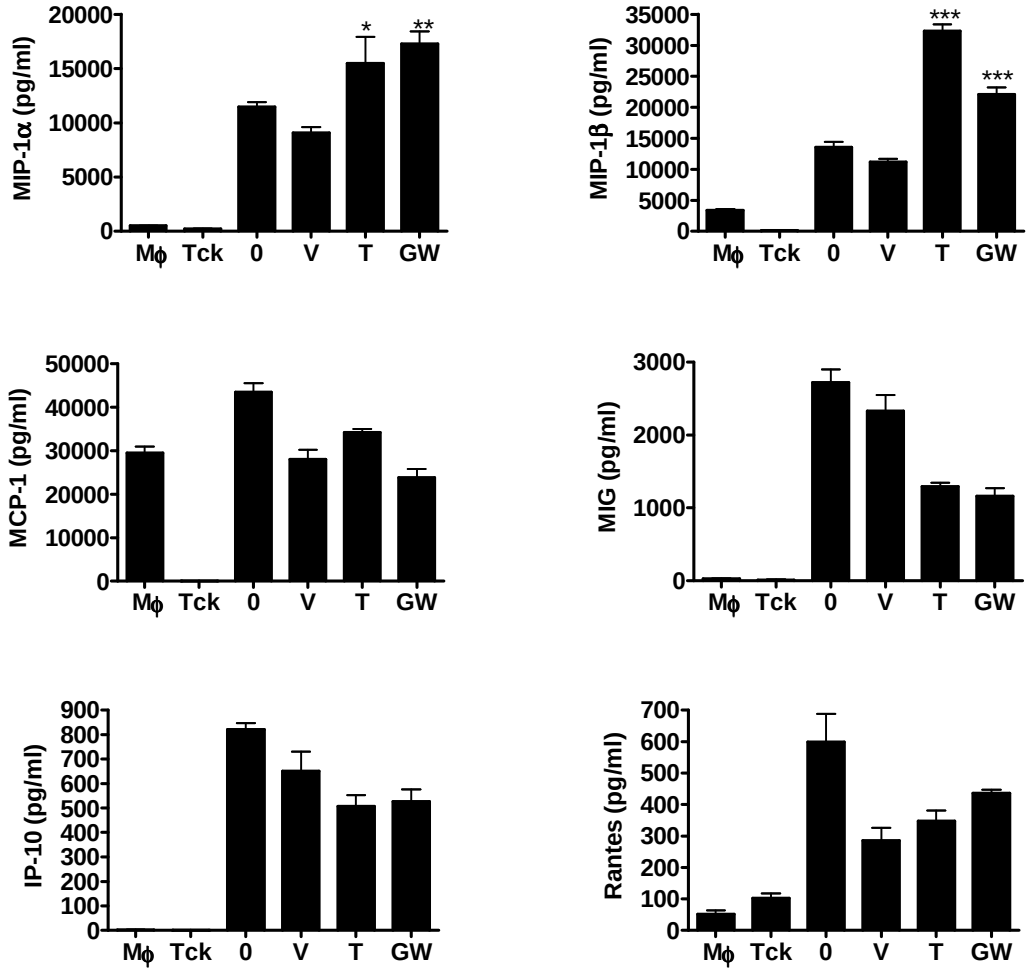


Figure 10.20 LXR agonism promotes inflammatory chemokine secretion from T cell activated macrophages.
Primary human monocytes were purified and matured to a macrophages (MΦ) phenotype with MCSF. Simultaneously, syngeneic $CD3^+$ T cells were activated with IL-2, IL-6 and TNFα (cytokine activated T cells- TcKs). After seven days the cells were co-culture and incubated overnight in the presence of media alone (0), vehicle (V – DMSO), 4 μM T1317 (T) or 4 μM GW3965 (GW). The concentration of chemokines was measured by Luminex. Each condition was tested in triplicate. Students T test; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

10.2.6 The effects of LXR activation are contact mediated

The precise mechanism(s) by which TcKs can induce macrophage activation is (are) not fully understood although several ligand pairs have been implicated e.g. CD40/ CD40L, ICAM1 / LFA-1 and CD45/CD45R (309, 310). Furthermore, how LXRs interact with such inflammatory pathways to promote macrophage cytokine and chemokine secretion is unknown. To determine whether the effect of LXRs was mediated through soluble mediators or via mechanisms that support cell surface interactions human macrophages and TcKs were generated as previously described. The TcKs were then placed either in co-culture with the macrophages

or in the same well but on a transwell insert, to keep the T cells and macrophages spatially separated. Cells were treated with media alone, vehicle or T1317 (Figure 10.21). Transwell inserts with a pore size of 0.4 μM were selected so as to allow the diffusion of soluble proteins but not intact cells. As expected, co-culture of MCSF matured macrophages with TcKs induced the secretion of TNFα which was increased by approximately two fold by treatment with T1317; supporting previous findings suggesting a pro-inflammatory effect of LXR agonism. However, when the TcKs were separate from the macrophages TNFα was not detectable and addition of T1317 mediated no effect. Similarly, pre-treatment of only the TcKs, but not the macrophages, with LXR agonist during the cytokine activation stage had no effect upon subsequent TNFα secretion (Figure 10.22). These data suggest that the mechanism by which LXR agonism potentiates TcK induced macrophage derived inflammatory cytokine secretion is mediated through enhancement of cell-cell surface interactions rather than via elaboration of soluble mediators and that LXR activation in both the TcKS and macrophages is required to increase the secretion of TNFα.

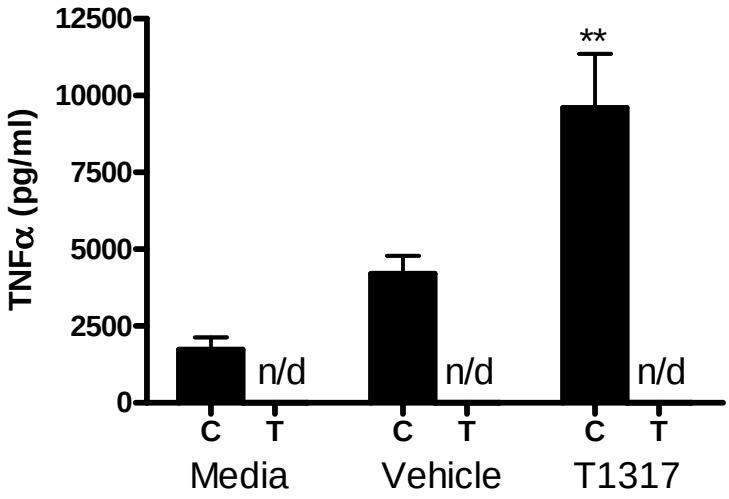


Figure 10.21 LXR agonism potentiates T cell induced macrophage derived pro-inflammatory cytokine secretion through cell surface interactions.
Human macrophages and cytokine activated T cells were co-cultured (C) or were physically separated by transwell inserts (T) and treated with media, vehicle (DMSO) or 4 μM T1317. The mean concentration of TNFα was measured by ELISA; not detectable (n/d). Each condition was tested in triplicate. Representative results from three individual donors. Students T test; ** $P \leq 0.01$.

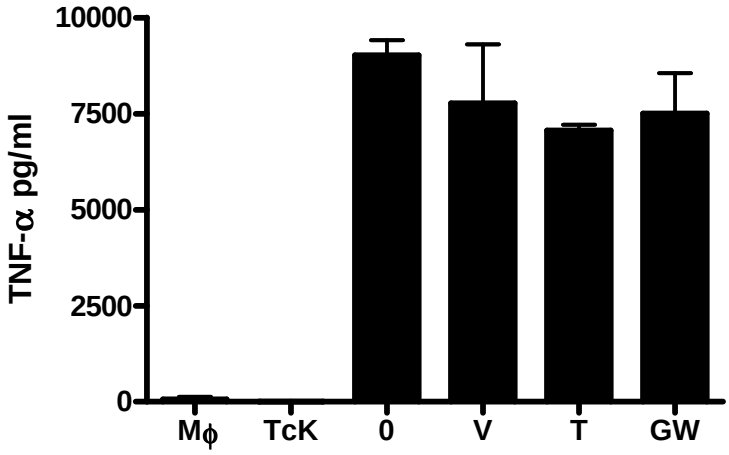


Figure 10.22 LXR agonism in both TcKs and macrophages is required to potentiate the secretion of TNFα.
Human CD14$^+$ monocytes were matured to a macrophage (MΦ) phenotype with MCSF whilst syngeneic T cells were cytokine activated (TcKs). The T cells, but not the macrophages, were pre- treated with media alone (0), vehicle (V- DMSO) 4 μM T1317 (T) or 4 μM GW3965 (GW) prior to being co-cultured with macrophages. The concentration of TNFα was measured by ELISA.

10.2.7 TNFα regulates the expression of LXRs

10.2.7.1 TNFα induces the expression of LXRα expression in murine macrophages

Several reports suggest a cell type / subset specific capacity for TNFα to modulate expression of LXRα and the LXR target gene ABCA1. Specifically, the expression of LXRα is increased by TNFα in rabbit adipocytes whereas in mouse and human kidney and liver cells the expression of LXRα is reduced (40, 41, 44). However, the effect of TNFα upon LXRα expression in macrophages is unknown.

Therefore, to determine if TNFα could modulate the expression of LXRα in macrophages, BMDM were treated with 5 ng/ml TNFα; a concentration which was previously shown to modulate LXRα expression in murine adipocytes (Figure 10.23) (44). The macrophages were incubated with TNFα for 4 hrs, 8 hrs, 12 hrs or 24 hrs to determine whether any effect of TNFα upon LXRα expression was a primary or secondary transcriptional event. Treatment of murine macrophages with TNFα significantly increased the expression of LXRα by approximately two fold at all time points indicating that the effect of TNFα upon LXRα expression was likely a primary transcriptional event. Previous reports have demonstrated

that TNFα can alter the expression of LXRα but not LXRß. BMDM were therefore treated with 2.5 ng/ml to 20 ng/ml TNFα for 24 hrs to firstly determine the effect of TNFα upon the expression of LXRß and LXR target genes and to secondly determine the optimal concentration of TNFα (Figure 10.24). The expression of LXRα was significantly increased by all concentrations of TNFα- similar to the previous experiment treatment, 5 ng/ml TNFα increased the expression of LXRα by approximately two fold. Similarly, the expression of ABCA1 was significantly increased by distinct concentrations of TNFα. However, although the expression of LXRß was significantly increased by treatment with 2.5 ng/ml and 10 ng/ml TNFα these effects were modest.

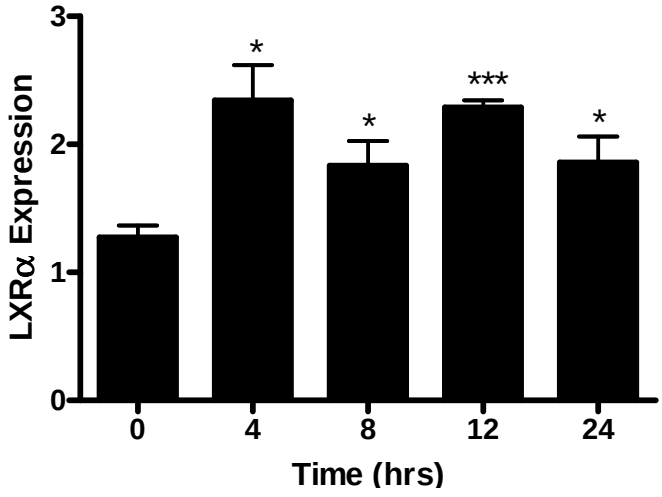


Figure 10.23 TNFα up-regulates the expression of LXRα in murine macrophages. Murine bone marrow derived macrophages were treated with 5 ng/ml TNFα for the indicated period of time. RNA was extracted for the analysis of LXRα expression by Taqman QRT-PCR and normalised to TATA binding protein (TBP). n = 4/ group. Students T test; * P ≤ 0.05, *** P ≤ 0.001.

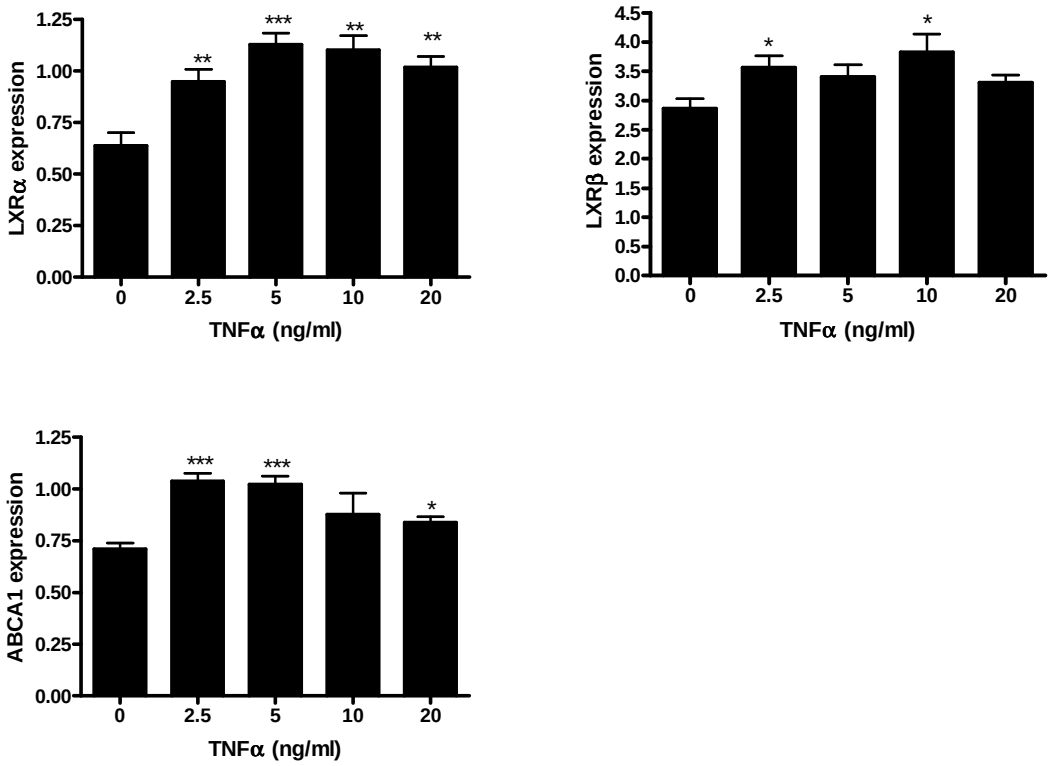


Figure 10.24 TNFα induces the expression of LXRα and downstream LXR target genes. Murine bone marrow derived macrophages were treated with TNFα at the indicated concentrations for 24 hrs. RNA was extracted for mean gene expression analysis of LXRα, LXRβ and ABCA1 by Taqman QRT-PCR normalised to TATA binding protein (TBP). Each condition was tested in triplicate; n = 5/ group. Students T test; * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001.

10.2.7.2 TNFα but not IL-6 induces the expression of LXRα in human macrophages

TNFα can induce the expression of LXRα and subsequently ABCA1 in murine macrophages. Similar effects upon LXRα expression have also been shown by the administration of IL-1 and LPS to human liver cell lines and primary monocytes respectively (40, 311). The effect of TNFα and other inflammatory cytokines upon the expression and activation of LXRs in primary human monocyte derived macrophages was unknown. Therefore, MCSF matured human macrophages were treated with increasing concentrations of TNFα or IL-6 for twenty four hours after which the expression of LXRα, LXRß and ABCA1 was measured by Taqman QRT-PCR (Figure 10.25). The concentration range of TNFα was derived from previous work in the literature and comparable to concentrations used in murine macrophages in previous experiments (Figure 10.24) (40, 41). The effect of IL-6 upon LXR expression has not previously been assessed. IL-6 is effective *in vitro* at 100 ng/ml – for example at this concentration it efficiently promotes T cell

activation. Treatment with TNFα significantly increased the expression of LXRα but not LXRß or ABCA1; P = 0.2556 and P = 0.6827 both at concentrations of 12.5 ng/ml respectively. Treatment with IL-6 did not affect the expression of LXRα, LXRß or ABCA1. These results suggest that TNFα but not IL-6 mediated signalling pathways are capable of modulating LXRα expression in human macrophages.

I next wished to analyse the down stream signal pathways that might explain these observations. TNFα binding to the TNF receptor (TNFR1) induces the recruitment of the adaptor molecule TRADD which subsequently recruits the signalling molecules TRAF2 and TRAF5 leading to activation of NF-κB (reviewed in (42)). Similarly, IL-1 and LPS have also been shown to exert modulatory effects upon the expression of LXRα both of which ultimately induce the activation of NF-κB (40, 311, 312). Both human and murine LXRα contain NF-κB response elements within their promoter region proximal to the transcriptional start site (Figure 10.26). To determine if the effects of TNFα upon LXRα expression were mediated through NF-κB activation murine BMDM were treated with 5 ng/ml TNFα and a pharmacological inhibitor of NF-κB, Bay11-7082 (Bay11), at concentrations between 2.5 μM and 20 μM or vehicle (DMSO) (Figure 10.27). Concentrations of Bay11 were selected from previous work in the literature which was shown to inhibit NF-κB induced expression of E-selectin at an Ec50 of 10 μM (313). Similar to previous experiments TNFα significantly increased the expression of LXRα in human macrophages by approximately four fold. Addition of Bay11 significantly reduced the expression of LXRα below basal levels of LXRα expression.

Further control experiments were essential to interpret this observation. NF-κB activation promotes cell survival and suppresses apoptosis therefore inhibition of NF-κB may potentially promote macrophage apoptosis. Therefore, to ensure that the macrophages were viable after treatment with Bay 11 BMDM were treated with vehicle (DMSO) or 2.5 μM Bay11 for 4 hours and analysed by flow cytometry. To assess cell viability after treatment with BAY11 the cells were stained with Propidium Iodide (PI) (Figure 10.28). PI stains DNA but can only cross the plasma membrane of non-viable cells; therefore PI positive cells are considered apoptotic or necrotic. Treatment of BMDM with BAY11 significantly increased the percentage of PI positive cells compared to vehicle suggesting that inhibition of NF-κB by BAY11 induced macrophage apoptosis. Therefore, although the

expression of LXRα was reduced in macrophages treated with BAY11 it is not clear if this is due to the induction of apoptotic or necrotic pathways or specific inhibition of NF-κB mediated induction of LXRα expression. Further studies utilising LXRα luciferase reporters are therefore required to confirm that the effect of TNFα upon LXRα expression is mediated through NF-κB. Time availability in my thesis studies precluded my performing these experiments.

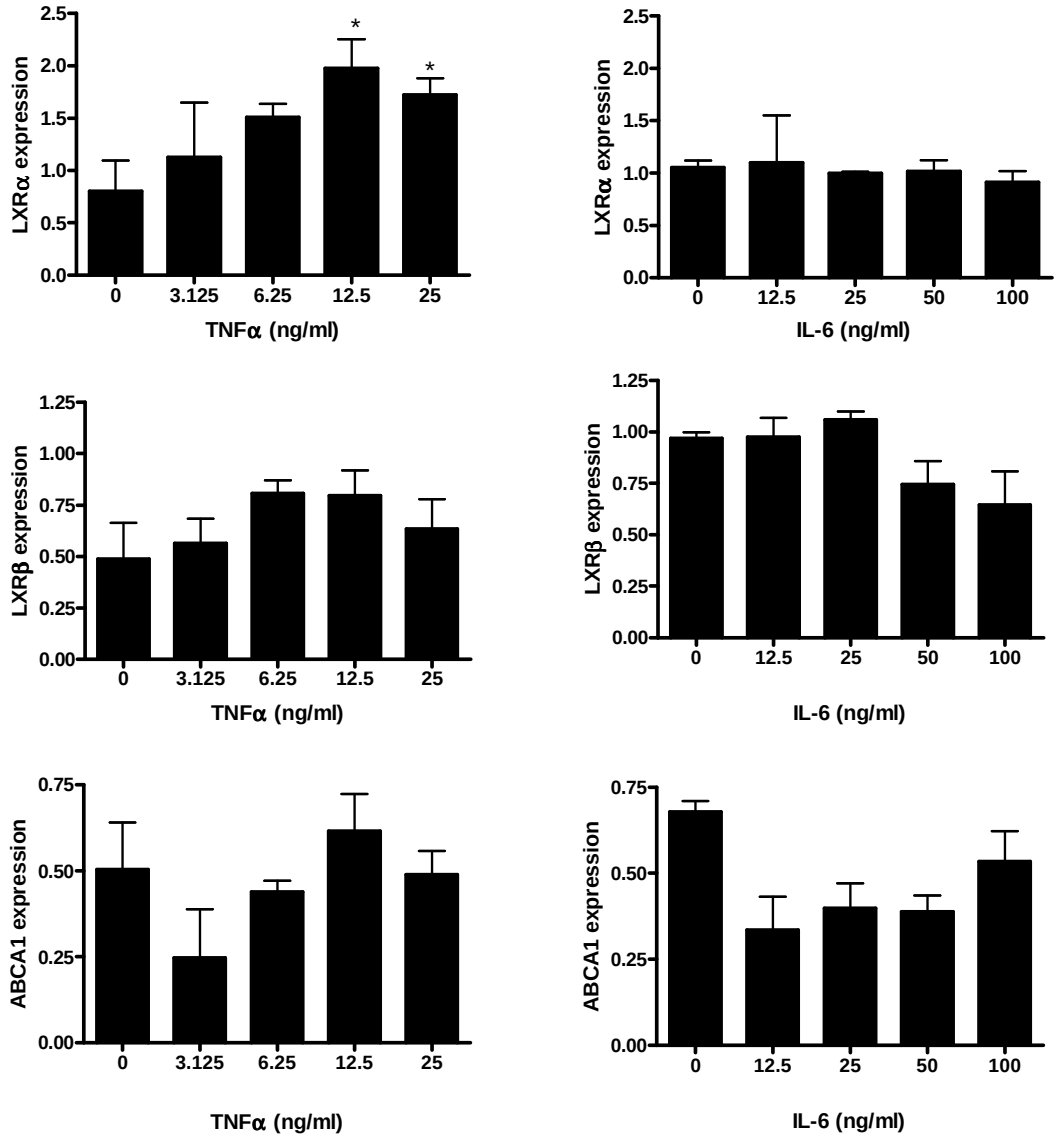
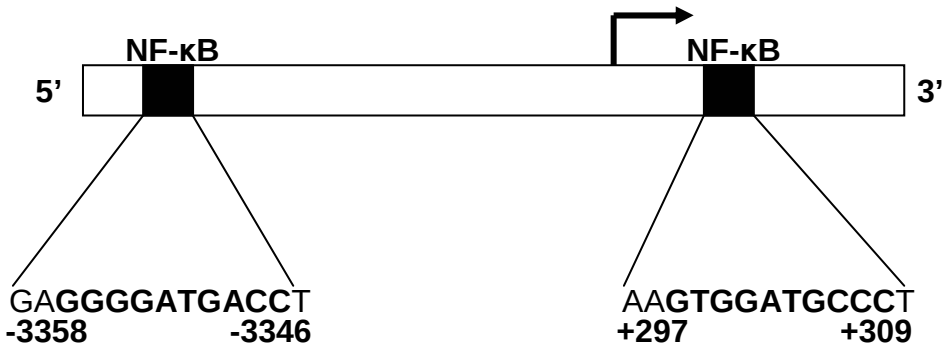


Figure 10.25 TNFα induces the expression of LXRα in human macrophages.
MCSF matured human macrophages were treated with TNFα or IL-6 at the indicated concentrations for 24 hrs. RNA was then extracted for gene expression analysis of LXRα, LXRβ and ABCA1 measured by Taqman QRT-PCR and normalised to TATA binding protein (TBP). Each condition was tested in triplicate and the results are representative of four individual donors. Students T test; * $P \leq 0.05$.

Mouse



Human

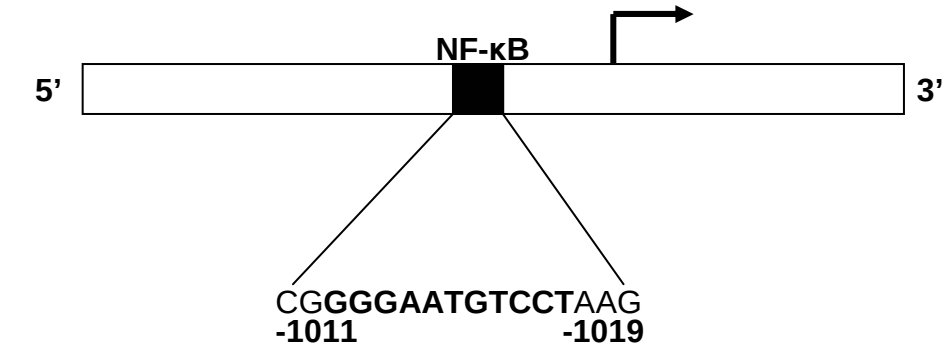


Figure 10.26 The human and mouse LXRα promoters contain NF-κB response elements. A schematic demonstrating the location of the NF-κB binding sites within the human and mouse LXRα promoters. The sequence of the NF-κB binding element is shown in bold (5’ to 3’) and the position relative to the transcriptional start site in base pairs is shown below. The transcriptional start site is indicated by the arrow.

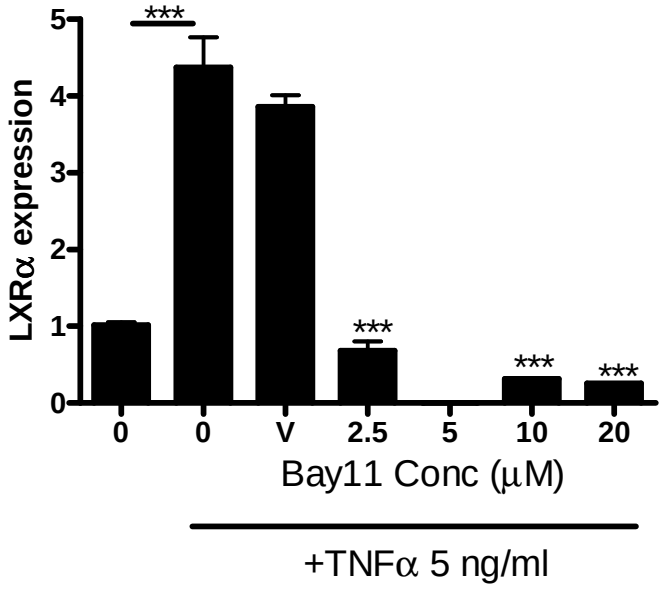


Figure 10.27 Inhibition of NF-κB activation reduces the expression of LXRα.
Murine bone marrow derived macrophages were treated with 5 ng/ml TNFα and the pharmacological NF-κB inhibitor Bay11-7082 (Bay11) or vehicle (V- DMSO) for 24 hrs. RNA was extracted for gene expression analysis of LXRα, normalised to TATA binding protein (TBP) by Taqman QRT-PCR. Each condition was tested in triplicate; n = 4/ group. Students T test; *** P ≤ 0.001.

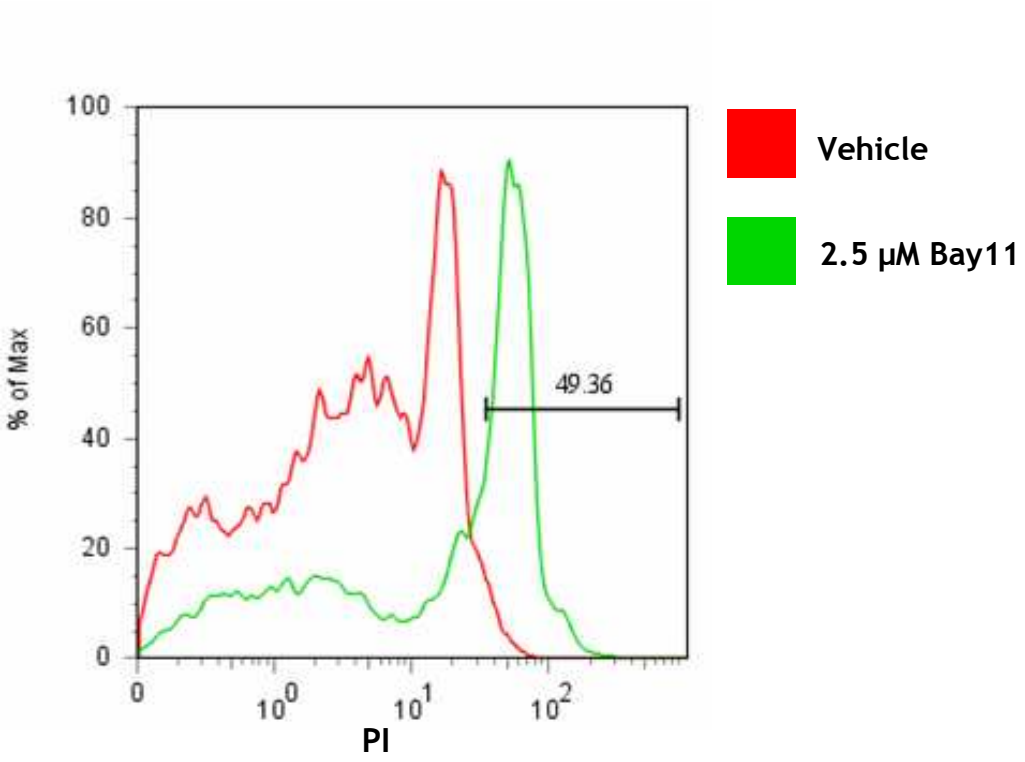


Figure 10.28 Inhibition of NF-κB by BAY11 induced macrophage apoptosis.
Murine bone marrow derived macrophages were treated with vehicle (DMSO) or 2.5 μM BAY11 for 4 hrs. The cells were then stained with Propidium Iodide (PI) to assess cell viability. Representative results of n = 3 mice.

10.2.8 Characterisation of LXR expression in human inflammatory arthropathies

10.2.8.1 Expression of LXRs is increased in PBMCs from AS patients

In addition to the maintenance of a cholesterol homeostasis it is now well established that LXRs play an immuno-modulatory role - my data discussed thus far suggest a predominantly pro-inflammatory effect upon LXR activation. Although the role for LXRs in arthritis is unknown recent reports in the literature support a role for LXRs in human disease pathology in which the expression of LXRα and LXRß is altered in the PBMCs of patients with multiple sclerosis (MS) but not other neurological conditions (123, 124). Therefore, to determine if the expression of LXRs is altered in RA, PBMCs were purified from patients with RA and healthy controls (HC). For comparison with other inflammatory arthropathies, PBMCs were obtained from patients with ankylosing spondylitis (AS) and psoriatic arthritis (PsA) to determine whether any biology around LXR expression was specific to RA or common to other autoimmune inflammatory conditions. The number of subjects and patient characteristics is shown in Table 10.1; all patients had established disease, met ACR diagnostic criteria and were receiving DMARD therapy as prescribed by their physician. RNA was extracted from the PBMCs and the level of expression of LXRα, LXRß and ABCA1 was measured by Taqman QRT-PCR (Figure 10.29). There was no correlation of LXRα, LXRß or ABCA1 expression with age in any of the subject groups (Table 10.2). Therefore, differences in subject age could not account for any changes in the level of LXR expression.

The expression of LXRα was not altered in the patients with RA or PsA relative to HC. Unexpectedly, LXRα mRNA expression was significantly higher in AS patients by approximately twenty two fold. Similarly, the expression of LXRß was significantly higher in AS compared to HC by approximately seventeen fold. There was also a significant, but modest, increase in the expression of LXRß expression in PsA whilst in RA the expression of LXRß was significantly lower than HC. The expression of ABCA1 was measured to determine if increased LXR transcriptional activation was associated with increased LXR expression. However, the expression of ABCA1 was constant between all subject groups

tested. These data demonstrate that the expression of LXRs is increased and suggest dysregulation of pathways which mediate the expression of LXRs specifically in AS.

	HC	RA	PsA	AS
Samples	36	48	22	19
Sex: Male (%)	20 (56)	11 (23)	10 (45)	16(84)
Female (%)	16 (44)	37 (77)	12 (55)	3(16)
Age: Mean (range)	39 (22-64)	60 (35-84)	54 (35-67)	58 (39-73)
ESR: Mean (range)	N/A	14 (1-48)	12 (2-30)	12.7 (4-29)
CRP: Mean (range)	N/A	9.3 (0.5-109)	9.6 (1-35)	10.1 (0.8-44)

Table 10.1 Characterisitics of patients and healthy control subjects.
Healthy controls (HC), Rheumatoid arthritis (RA), psoriatic arthritis (PsA) and ankylosing spondylitis (AS). Data not available (N/A).

R^2 (P)	HC	RA	PsA	AS
LXRα	0.012 (0.52)	0.009 (0.47)	0.002 (0.82)	0.009 (0.76)
LXRβ	0.06 (0.64)	0.013 (0.39)	0.015 (0.59)	0.103 (0.08)
ABCA1	0.008 (0.6)	0.001 (0.86)	0.005 (0.75)	0.001 (0.93)

Table 10.2 The expression of LXRs does not correlate with age.
The expression of LXRα, LXRβ and ABCA1 was measured by Taqman QRT-PCR. Healthy controls (HC), Rheumatoid arthritis (RA), psoriatic arthritis (PsA) and ankylosing spondylitis (AS). Values shown are linear regression (r^2) and P value of significance in brackets.

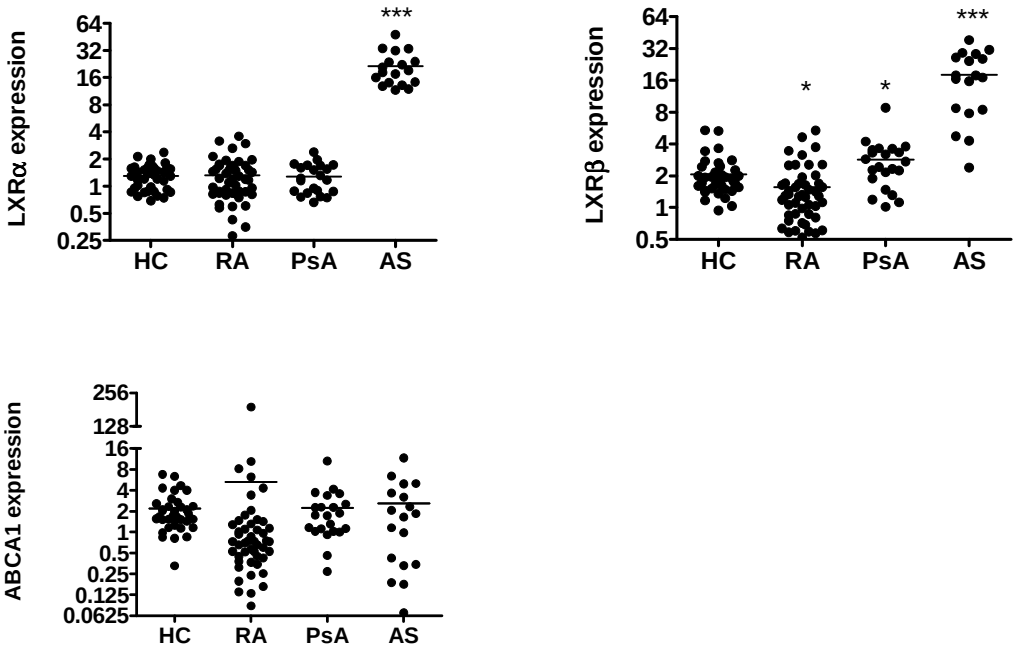


Figure 10.29 The expression of LXRs is higher in ankylosing spondylitis.
The expression of LXRα, LXRβ and ABCA1, normalised to TATA binding protein (TBP) was measured by Taqman QRT-PCR in peripheral blood mononuclear cells purified from healthy controls (HC) or patients with rheumatoid arthritis (RA), psoriatic arthritis (PsA) and ankylosing spondylitis (AS). Students T test; * P ≤ 0.05, *** P ≤ 0.001.

CRP/ ESR	RA	PsA	AS
LXRα	0.004 (0.67)/ 0.034 (0.24)	0.003 (0.82)/ 0.051 (0.31)	0.04 (0.43)/ 0.188 (0.07)
LXRß	0.062 (0.09)/ 0.141 (0.09)	0.004 (0.78)/ 0.141 (0.09)	0.025 (0.53)/ 0.012 (0.67)
ABCA1	0.238 (0.007)/ 0.212 (0.002)	0.002 (0.84)/ 0.075 (0.22)	0.139 (0.13)/ 0.003 (0.83)

Table 10.3 The expression of LXRs or ABCA1 does not correlate with disease severity. Linear regression analysis (r^2) of disease severity as measured by CRP and ESR in Rheumatoid arthritis (RA), psoriatic arthritis (PsA) and ankylosing spondylitis (AS); patient characteristics and numbers are shown in Table 10.1. The expression of LXRα, LXRβ and ABCA1 was measured by Taqman QRT-PCR. Values shown are linear regression of CRP/ ESR vs LXR or ABCA1 expression and P values are shown in brackets.

10.2.8.2 LXR mediated cytokine secretion is increased in patients with ankylosing spondylitis

I next wished to determine whether this elevated basal level of LXR expression is in PBMCs of AS patients had any functional significance. The increased expression of LXRs suggests that PBMCs from AS patients may potentially have an increased capacity to respond to LXR agonists and subsequently promote inflammation. I have previously shown that LXR agonism can increase LPS mediated secretion of pro-inflammatory cytokines/ chemokines from human primary monocytes (Figure 10.8) an observation which has since been confirmed by other studies in the literature (61). Therefore, to determine how increased expression of LXRs may impact the secretion of IL-6 in response to TLR4 ligation with LPS and LXR agonism PBMCs were purified from subjects with AS, PsA, RA and HC. The subject characteristics and numbers are described in Table 10.4. PBMCs were pre-incubated with media alone, vehicle (DMSO) or 0.25 μM to 4 μM

GW3965 for 36 hours prior to addition of 100 ng/ml LPS. After twenty four hours the concentration of IL-6 in the cell culture supernatants was measured by ELISA (Figure 10.30). Consistent with previous findings LXR activation significantly increased the secretion of IL-6 in response to LPS from PBMCs of HC in a dose responsive manner. Similarly, the concentration of IL-6 was also significantly increased from LPS stimulated PBMCs of PsA, RA and AS patients in response to LXR activation. However, the concentration of IL-6 secreted from AS PBMCs was significantly higher than that secreted from HC in response to TLR4 ligation at several concentrations of GW3965. Similarly, the concentration of IL-6 secreted from PsA PBMCs was significantly higher than HC but only at the highest concentration of 4 μM GW3965. There was no significant difference in the concentration of IL-6 secreted from RA PBMCs compared to HC (P = 0.6423). These data indicate that AS PBMCs have an increased capacity to respond to LXR agonists and subsequently promote an inflammatory response mediated by TLR4 stimulation with LPS.

	HC	**RA**	**PsA**	**AS**
Samples	12	11	10	16
Sex: Male (%)	8 (66)	5 (45)	3 (30)	11 (68)
Female (%)	4 (33)	6 (55)	7 (70)	5 (32)
Age: Mean (range)	43 (25 – 59)	60 (57-77)	46.6 (32-64)	48 (24-75)
ESR: Mean (range)	N/A	14.8 (2-56)	15.6 (1.5-57)	8.7 (1.8-25)
CRP: Mean (range)	N/A	15.6 (0.8-33)	13.4 (0.7-29)	9.9 (0.7-9.9)

Table 10.4 Subject characteristics.
Healthy controls (HC), Rheumatoid arthritis (RA), psoriatic arthritis (PsA) and ankylosing spondylitis (AS). Data not available (N/A).

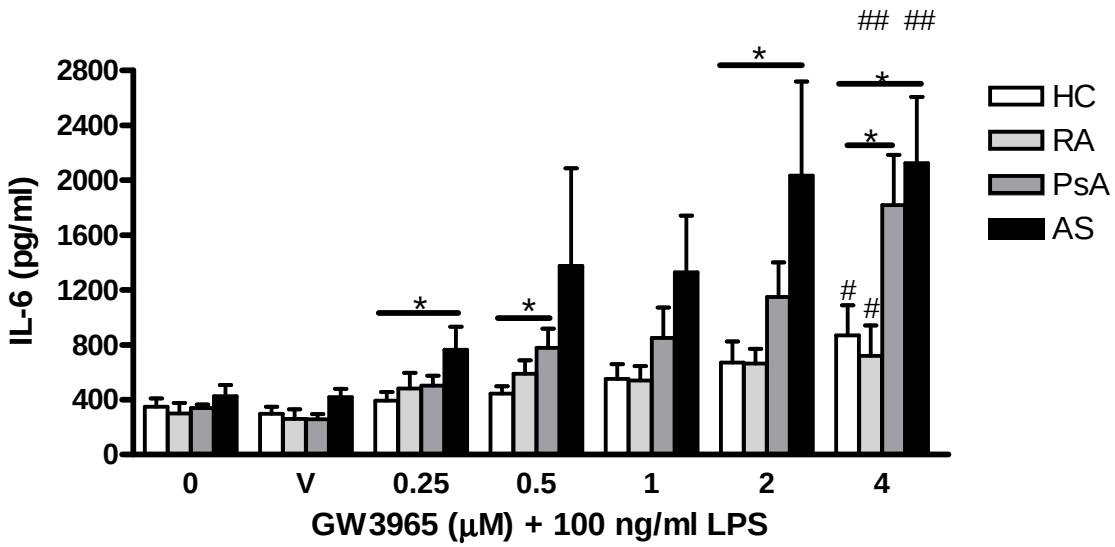


Figure 10.30 LXR mediated cytokine secretion is increased in patients with ankylosing spondylitis.
PBMCs were purified from healthy controls (HC), Rheumatoid arthritis (RA), psoriatic arthritis (PsA) and ankylosing spondylitis (AS) and pre-incubated with media alone (0), vehicle (V- DMSO) or GW3965 at the indicated concentrations for approximately 36 hrs. The PBMCs were then stimulated with 100 ng/ml LPS for 24 hrs and the concentration of IL-6 in the cell culture supernatants was measured by ELISA. The subject characteristics and numbers are shown in Table 10.4. Each condition was tested in triplicate. Students T test; * $P \leq 0.05$ between disease groups and healthy controls at the same concentration of GW3965 or # $P \leq 0.05$, ## $P \leq 0.01$ at concentrations of GW3965 within the same subject group relative to vehicle control.

10.3 Discussion & conclusion

There is now a large body of evidence supporting a role for LXRs in inflammation in which LXR activation is generally ascribed an anti-inflammatory effect. This was first demonstrated by the treatment of LPS stimulated murine macrophages with LXR agonists which inhibited the expression of iNOS (117). Similarly, my own and other studies in the literature have since confirmed these findings and have demonstrated similar inhibitory effects of LXR agonism upon LPS stimulation *in vivo* (19, 118, 314, 315). Together these studies suggest a general anti-inflammatory effect of LXR agonism in murine systems. In support of this, I found that the anti-inflammatory effect of LXR activation is not unique to TLR4 ligation but is also common to TLR2 induced inflammatory pathways as GW3965 reduced the secretion of IL-6 from murine macrophages stimulated with the TLR2 ligands PAM3Cys or LTA. However, it is unclear what effect activation of LXRs has upon other TLR signalling pathways as I was unable to detect IL-6 by ELISA in cell culture supernatants from TLR3, TLR7/8 or TLR9 stimulated murine macrophages. TLR3, TLR7, TLR8 and TLR9 predominantly sense viral infection through the binding of dsRNA and DNA to promote the secretion of IFNα or IFNß. Therefore, measurement of IFNα and IFNß concentrations in cell culture supernatants by ELISA are required to determine if LXR agonism can affect IFN secretion and thereby infer a potential role for LXRs in defence against viral infection.

Joseph *et al* previously demonstrated that the inhibitory effect of LXR agonism upon TLR4 induced iNOS expression was mediated by antagonism of NF-κB signalling (117). Similar to TLR4 ligation TLR2 also induces the activation of NF-κB. It is therefore likely that inhibition of TLR2 induced IL-6 secretion by LXR agonism is mediated through the inhibition of NF-κB signalling pathways. This remains to be formally tested - the use of an NF-κB luciferase reporter would inform any inhibitory effect of LXRs upon NF-κB activation. These results suggest that LXR activation would be detrimental towards the clearance of a bacterial infection. However, contradictory to this hypothesis mice lacking LXRs are highly susceptible to *Listeria monocytogenes* infection and exhibit defective bacterial clearance *in vivo* (316). This was mediated by accelerated macrophage apoptosis due to aberrant regulation of the anti-apoptotic factor SPalpha; I conclude at

this stage only that the role of LXRs in innate immunity is complex and requires further investigation. I am also struck by the distinctions between murine and human studies thus far and interpolate cautious interpretation of my own in vivo experiments that constituted part of my own volume of work herein.

The role of LXRs in inflammation has been predominantly studied in rodents or rodent derived tissues. Therefore, how LXRs modulate an inflammatory response in human cells was until recently unknown. I therefore first sought to determine if the anti-inflammatory effect of LXR agonism upon TLR4 induced inflammatory pathways in murine macrophages was conserved in humans. Paradoxically, treatment of human LPS stimulated monocytes with LXR agonists markedly increased the secretion of multiple pro-inflammatory cytokines and chemokines. These results demonstrate a novel pro-inflammatory effect of LXR activation in humans and are in direct contrast to the effect of LXR agonism in mice. Fontaine *et al* have since confirmed these results and demonstrated that the pro-inflammatory effect of LXR agonism upon TLR4 activation is through the increased expression of TLR4 mediated by LXRα (61). Stimulation of TLR4 with LPS induces the phosphorylation of the MAPKs Jnk, Erk and p38 and accordingly LXR agonism increases MAPK phosphorylation downstream of TLR4. These data suggest that LXR activation potentiates the inflammatory response through increased TLR4 expression and downstream signalling pathways leading to enhanced pro-inflammatory cytokine secretion. Notably, the LXRE is not present within the murine TLR4 promoter hence LXR activation in murine macrophages does not alter TLR4 expression. Additionally, Fontaine *et al* also demonstrated a biphasic effect of LXR activation in human macrophages. Pre-treatment of human LPS stimulated macrophages with LXR agonists for 12 hrs or less decreased the secretion of MCP-1 and TNFα whereas MCP-1 and TNFα secretion was increased from macrophages pre-incubated with LXR agonists for 24hrs or longer. These observations are in agreement with my results where monocytes were pre-incubated with T1317 or GW3965 for 24 hrs or longer.

The induction of human TLR4 expression by LXRα is a primary transcriptional event, evidenced by the direct binding of LXRs to the TLR4 promoter. However, the expression of human TLR4 is only increased in macrophages pre-incubated with LXR agonists for longer than 24 hrs, hence cytokine secretion is only increased in monocyte/ macrophages pre-incubated with LXR agonist for longer

than 24 hrs. Although no change in TLR4 expression is observed with shorter periods of macrophage/ LXR agonist pre-incubation, it is evident that there is an inhibitory effect of LXR agonism upon inflammatory cytokine/ chemokine secretion. It is not clear how LXRs mediate the switch between the biphasic effects upon the regulation of TLR4 mediated signalling pathways. However, LXRs have been shown to promote the expression of the nuclear receptor Rev-erbα which inhibits TLR4 expression (5). Furthermore, whether LXR agonism in human monocytes/ macrophages inhibits NF-κB activation similar to that demonstrated in murine macrophages at the earlier time points of agonist incubation is unknown. This is summarised in Figure 10.31. The effect of LXR agonism upon other TLR mediated pathways in human macrophages has not been tested however these studies so far have demonstrated a novel pro-inflammatory effect of LXR agonism upon TLR4 mediated inflammation in human monocytes/ macrophages.

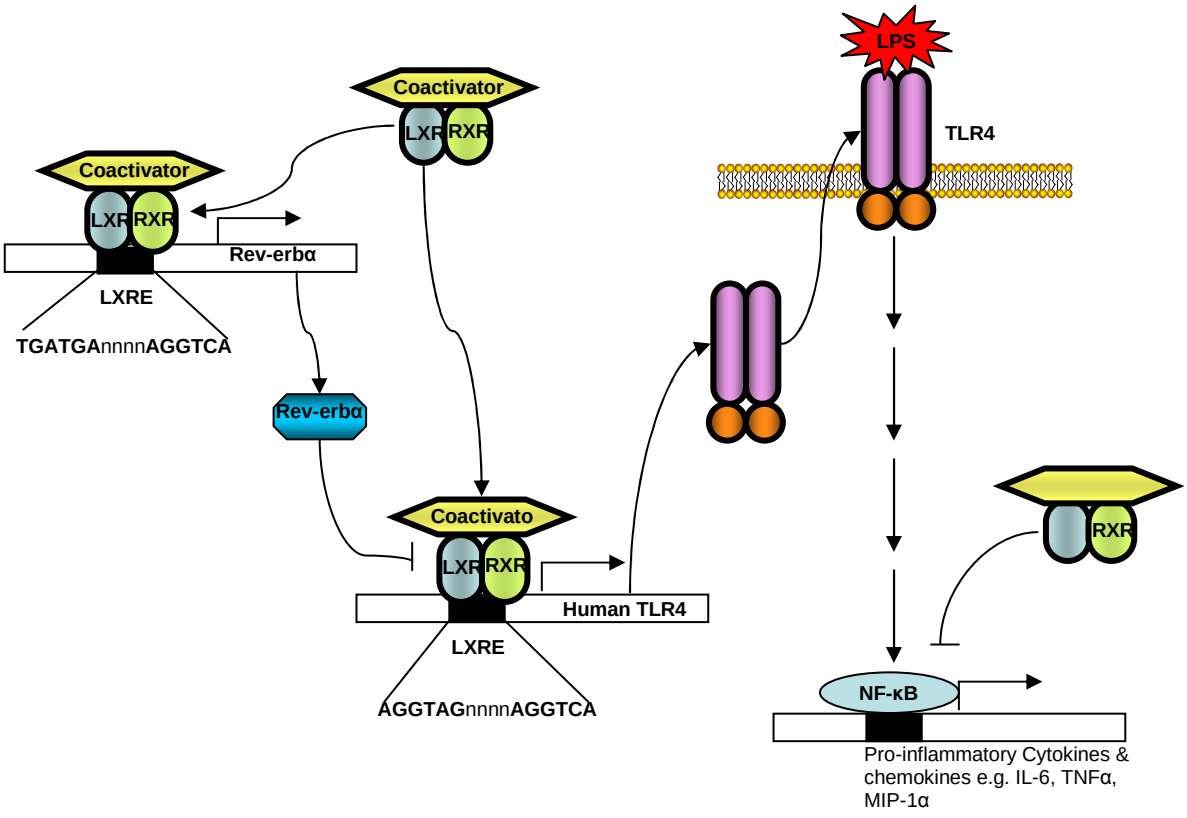


Figure 10.31 Regulation of TLR4 expression and signalling pathways by LXRs. Activation of LXRs is able to upregulate the expression of TLR4 in human macrophages leading to increased downstream signalling and secretion of inflammatory cytokines and chemokines. However, LXRα can also induce the expression of Rev-erbα which can inhibit LXR mediated induction of TLR4; thereby negative feedback upon TLR4 expression. In murine macrophages LXR can also inhibit the activation of NF-κB and the expression of downstream target genes it is not clear if there is any interaction between LXR and NF-κB signalling pathways in human macrophages.

The pro-inflammatory effect of LXR agonism upon TLR4 induced cytokine secretion from human peripheral blood derived monocytes is conserved in patients with RA. LXR agonism may therefore support the development of an inflammatory environment through the secretion of cytokines and chemokines induced via the activation of TLR4 by either endogenous or bacterial derived ligands. Unlike monocytes/ macrophages treatment of fibroblasts or synoviocytes with T1317 or GW3965 did not change the basal or likewise the concentration of LPS induced secretion of TNFα or IL-6. This demonstrates that fibroblasts and synoviocytes are not already maximally activated as addition of LPS increased the secretion of IL-6. Secondly these results suggest that the pro-inflammatory effect of LXR may potentially be exclusive to the periphery.

In support of a role for LXRs in driving inflammation in the synovium LXR activation increased the secretion of multiple macrophage derived pro-inflammatory cytokines and chemokines in the TcK/ macrophage contact assay. By using a transwell assay system I was able to confirm previous findings demonstrating that the secretion of TNFα was mediated through macrophage/ TcK cell-cell contact (162). As addition of T1317 did not affect the secretion of TNFα from macrophages cultured separately from TcKS this would suggest that LXRs were not able to induce the secretion of soluble mediators that were able to initiate the inflammatory response. Therefore, how LXR agonism drives macrophage derived pro-inflammatory cytokine secretion in this context is unknown but may be in part through positive feedback of IL-2, IL-6 and TNFα or IL-15 upon TcK activation.

To explore the role of potential feedback loops upon LXR biology, macrophages were treated with TNFα or IL-6. Interestingly, TNFα increased the expression of LXRα in both human and murine macrophages. Although the expression of LXRα was consistently increased it was not clear what effect TNFα exerted upon LXR transcriptional activation as ABCA1 was increased in murine but not human macrophages; the effect on other LXR target genes was not tested. These results have important implications for the role of LXRs in inflammation especially in RA where TNFα is central to disease pathology. My results have demonstrated a predominantly pro-inflammatory effect of LXR activation. This may be positively regulated by TNFα through the increased expression of LXRα thereby further enhancing the inflammatory response mediated by agonism of LXRs. It would

therefore be interesting to test the combined effect of TNFα with LXR agonists upon the secretion of inflammatory cytokines. Furthermore, if TNFα is able to increase the basal level of LXR transcriptional activation; as suggested by the increased expression of ABCA1, this would infer that TNFα is able to increase signalling pathways which support oxysterol synthesis or cellular uptake. Gas chromatography could be used to measure changes in endogenous oxysterol concentrations in response to treatment with TNFα to determine if TNFα mediates oxysterol synthesis and cholesterol metabolism. Similarly, the concentration of intra-cellular vs extra cellular LDL can be measured in macrophages treated with acetylated LDL to inform whether TNFα affects cholesterol uptake. These findings are summarised in Figure 10.32.

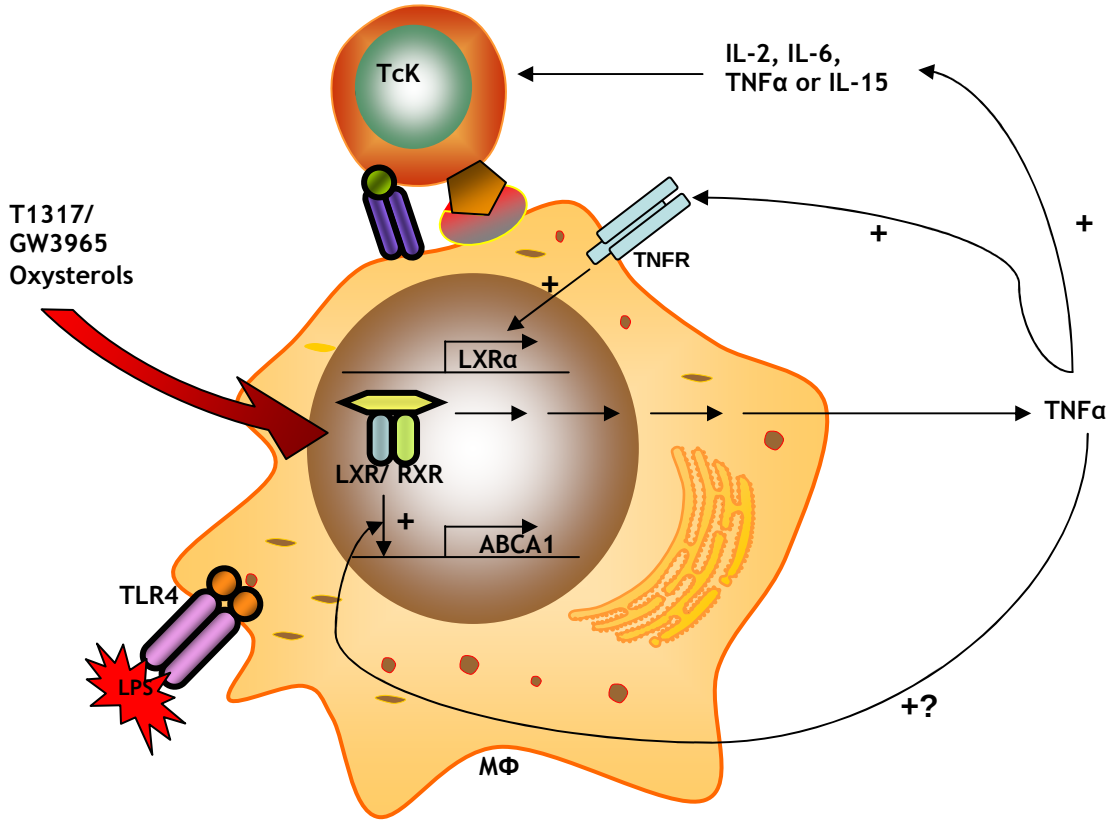


Figure 10.32 TNFα positively regulates LXRα expression.
LXR agonism increases the secretion of TNFα in response to TLR4 ligation or TcK induced activation of macrophages (MΦ) by an unknown mechanism. Increased concentrations of TNFα may positively regulate LXRα expression by signaling through the TNF receptor (TNFR). TNFα may also positively regulate the transcriptional activation of LXRs as suggested by the increased expression of ABCA1 in murine macrophages. The increased secretion of TNFα by LXR agonism may support TcK activation and thereby increase TcK induced activation of macrophages within the synovium.

Together with the pro-inflammatory effects of LXR agonism *in vivo* and *in vitro* and the potential positive feedback loops of TNFα upon LXRα expression, and potentially LXR activation, these results suggest that LXRs may play a role in RA disease pathology. Gene expression analysis of LXRα, LXRβ and ABCA1 showed that the expression of LXRs in RA PBMCs was similar to that of healthy controls. However, these results do not exclude a role for LXRs in RA pathology as LXRs are constitutively expressed and are activated by the binding of cholesterol derived oxysterol ligands therefore, basal levels of LXR expression, but increased LXR activation, may drive inflammation in RA. It is also likely that the mechanisms which lead to pathology in RA or PsA compared to AS are different and may exert distinct effects upon LXRs.

The expression of LXRs in RA PBMCs, as well as healthy controls, was also compared to that of patients with the spectrum of spondyloarthritis, namely psoriatic arthritis (PsA) and ankylosing spondylitis (AS). The expression of LXRα and LXRβ was dramatically increased in AS and moderately so in PsA. Both AS and PsA are autoimmune inflammatory conditions collectively affecting approximately 4% of the population. AS is manifest as inflammation of the sacroiliac joints and other enthesis organs, leading to fusion of vertebrae and curvature of the spinal column. Whereas PsA can have spinal involvement it is associated primarily with synovial and enthesial inflammation in peripheral joints and then with articular destruction. It is normally also associated with psoriatic skin lesions. Both conditions are distinct from RA by being seronegative for RF and ACPA. Although the pathogenesis of AS and PsA is unknown both conditions share some common genetic risk factors. SNPs in the IL-23 receptor and ERAP1 (ARTS-1) have been identified and are both associated with AS and PsA disease (317-321). HLA-B27 is also predictive of erosive arthritis in PsA and is present in approximately >90% of AS patients (322). The role of HLA-B27 in PsA and AS disease pathology is not full understood however several reports have suggested that HLA-B27 misfolding may trigger the unfolded protein response (UPR) causing endoplasmic reticulum (ER) stress (323, 324). Indeed, all the AS patients were HLA-B27 positive but the HLA-haplotype of the PsA patients was unknown.

A role for LXRs in ER stress has not yet been identified although, activation of PPARγ, which can induce the expression of LXRs, has been shown to induce ER

stress and attenuate subsequent IL-1 and IFNγ signalling (49, 325). Additionally, the transcription factor SREBP-1c, an LXR target gene, is present within the ER lumen. ER stress induces the cleavage of SREBP-1c and stabilises its binding to the promoters of target genes (326, 327). These observations implicate the LXR signalling pathway in ER stress but how ER stress may potentially effect LXR expression/ activation is unknown. It would therefore be of interest to further explore the association of HLA-B27 with LXR expression and determine how LXRs may effect inflammatory cytokine secretion in a context of ER stress. In addition to the genetic risk AS and PsA are associated with inflammatory bowel disease (IBD); ulcerative colitis and Crohn's disease. However, as IBD is only present in approximately 60% of AS and PsA patients it is unlikely that IBD is the reason for the increased expression of LXRs in all the PsA and AS samples tested; although the IBD status of these individuals was unknown. Whether the expression of LXRs may serve as a suitable biomarker for the development of AS is currently unknown but further studies are required to identify the mechanisms leading to the increased expression of LXRs specifically in AS.

11 Discussion & conclusion

At the outset of my studies, some reports suggested an immuno-modulatory role of LXR activation. I therefore sought to determine how activation of LXRs could impact upon inflammatory signalling pathways with potential consequences for RA disease pathology. Unexpectedly, I have demonstrated a profound and novel pro-inflammatory effect of LXR agonism. This is exemplified by the dramatic increase in articular inflammation and destruction observed with administration of T1317 or GW3965 *in vivo* in a murine model of CIA. Enhanced disease severity was associated with elevated numbers of Th1 and Th17 cells and increased serum concentrations of multiple pro-inflammatory cytokines and inflammatory chemokines. Consistent with these observations the secretion of inflammatory cytokines was increased by LXR agonism regardless of mode of activation, namely via TLR4 (LPS) or cell-cell interactions; TcK stimulated human macrophages. Overall, my results have demonstrated a previously unrecognised pro-inflammatory effect of LXR activation that may drive pathology and disease severity in arthritis.

My results contradict previous findings in the literature in which LXR activation has been described to exert a general anti-inflammatory effect. Many such studies have used murine models of disease or rodent derived tissue *ex vivo* in which an inflammatory response has been initiated by the use of LPS (19, 117, 118, 120, 314, 315, 328-331). In this context LXR agonism does indeed reduce the secretion of pro-inflammatory cytokines and ameliorates disease pathology induced by LPS. However, paradoxically LXR agonism in human LPS stimulated monocytes/ macrophages supports the secretion of pro-inflammatory cytokines (61). Therefore, studies in which inflammation is induced with LPS in rodents or rodent derived tissue are unlikely to accurately reflect the action of LXRs in human cells; neither do they inform the action of LXRs upon other inflammatory pathways. This is best exemplified where LXR agonism was shown to inhibit LPS induced airways inflammation in mice whereas administration of GW3965 in an LPS free model of asthma is clearly detrimental – thus LXR agonism in such a context in mice is pro-inflammatory (19, 119, 315). Likewise LXR activation has also been shown to induce the expression of inflammatory markers; ICAM-1, VCAM-1 and E-selectin, on human endothelial cells (105).

LXRs are best known and investigated for their role in lipid regulation and transport and therefore separating out whether the effects LXR agonism upon

inflammation is mediated directly or is a result of altered lipid metabolism may prove problematic. It is evident that LXRs can exert direct effects upon inflammatory signalling pathways as mediated by the increased expression of human TLR4 induced by LXRα (61). In support of a direct effect and as a potential explanation for the pro-inflammatory effects of LXR agonism in the murine CIA model, LXRs have been shown to regulate the expression of genes involved in glucocorticoid biology. GCs are synthesised and secreted in an inert form from the adrenal glands under the control of the hypothalamic-pituitary-adrenal (HPA) axis. Activation of GCs is mediated by the enzyme 11ß-hydroxysteroid-dehydrogenase-type-1 (11ßHSD-1) that catalyses the conversion of inert cortisone to active cortisol in humans permitting GC signalling through the glucocorticoid receptor (GR). Steroid signalling, as in the case of RA patients treated with for example prednisolone or triamcinolone hexanoate, exerts a profound anti-inflammatory effect which is mediated by increased T cell apoptosis and macrophage phagocytosis of apoptotic leukocytes (265, 266). LXR activation has been shown to inhibit the expression of 11ßHSD-1 and GR thereby reducing GC signalling and potentially the beneficial anti-inflammatory effects attributable GC (7, 8, 93). In accordance with an inhibitory effect upon GC enhanced disease severity was associated with increased lymph node T cell numbers and neutrophil accumulation; potentially mediated through decreased macrophage phagocytosis. Further studies are therefore required to elucidate how the action of LXRs may impact upon GC/ GR signalling to drive disease pathology in RA.

Part of my original interest in LXR biology came from the well recognised association of RA with enhanced vascular risk –indeed I considered at outset the possibility that LXR agonism might mediate beneficial effects in both tissue compartments namely primary articular disease and comorbid vascular atheroma. It is interesting to note that RA patients have accelerated CVD characterised by an atherosclerotic lipid profile and the presence of atherosclerotic lesions up to fifteen years prior to the clinical onset of RA (250, 251, 253, 257). It is now widely accepted that pathology associated with atherosclerosis and RA may cooperate to promote a state of generally enhanced inflammation. As well as understanding a role of LXRs in inflammation this study was also initiated in an attempt to understand how altered lipid metabolism may

drive RA pathology. I hypothesise that cholesterol degradation within the periphery to form oxysterols could drive the development of synovial pathology by activation of LXRs. Indeed, my results which suggest a pro-inflammatory effect of LXR activation support this hypothesis. However, although my studies, as with other reports in the literature using T1317 or GW3965, are informative of LXR activation they may not faithfully represent the action of specific endogenous oxysterol species upon the activation of LXRs. Synthetic LXR agonists induce activation of LXRs systemically whereas endogenous agonists are restricted to particular anatomical locations and indeed different oxysterols are recognised to differentially activate or inhibit LXRs (29). Further studies are therefore required to demonstrate that specific oxysterols do exert pro-inflammatory effects. Identifying which species of oxysterols are present in the serum and synovium of RA patients and if concentrations of these are altered relative to healthy controls is also important for understanding whether LXRs have a pathological role in RA. It would also be of interest to analyse the oxysterol content in patients both prior and post development of clinical arthritis. Nevertheless, my results do suggest that elevated levels of cholesterol, or altered cholesterol metabolism, may exert detrimental effects upon the progression of RA by activation of LXRs. This is in agreement with clinical trials performed by the host laboratory in which the use of atorvastatin modified markers of cardiovascular risk surrogates and exerted anti-inflammatory effects in RA (290). Notably *in vivo* studies have suggested that some of the anti-inflammatory effects of statins may be independent of altered cholesterol concentrations (332).

Understanding the individual roles of LXRα and LXRß is central to understanding how lipid metabolism may impact upon inflammation and for the design of future therapeutics; for example it is already recognised that agonists designed specifically towards the activation of LXRß are more favourable than dual LXRα/ LXRß agonists for the treatment of atherosclerosis (101, 102). Furthermore, deletion of LXRα or LXRß is protective against the pro-inflammatory effects mediated by administration of GW3965 or T1317 in murine CIA and suggests that cooperation between the LXR isoforms is necessary to drive inflammation. The separate role(s) of LXRα and LXRß in inflammation is (are) unknown. It would be of interest to determine whether cooperation between LXRα and LXRß is also

required to drive inflammation in human cells e.g. in the T cell / macrophage contact assay of synovitis. Importantly these findings have considerable implications for the development of LXR agonists as future therapeutics in which LXR agonists are already in pre-clinical trials for the treatment of atherosclerosis (36, 126). My results suggest that dual LXRα/LXRB agonism especially in patients with an underlying autoimmune condition or bacterial infection may cause an adverse pro-inflammatory effect. The current strategy of pharmaceutical companies is to develop agonists specifically targeted towards LXRß. The use of LXRß specific agonists may avoid the pro-inflammatory effects attributable to dual LXRα/ LXRß agonism whilst preserving the beneficial effects upon the regression of atherosclerosis disease progression. However, endogenous oxysterol LXR ligands activate both LXRα and LXRß and together with synthetic LXRß agonists may exacerbate an inflammatory response over a prolonged period of time. Therefore, future LXRß specific agonists should be thoroughly tested and used with caution.

While the new evidence on the potential involvement of LXRs in RA is intriguing, major issues remain to be explored. Future studies must identify which mediators are responsible for the pro-inflammatory effects of LXRs and should elucidate why LXR agonism may exert either a pro or anti-inflammatory response in different contexts and indeed species. Finally, understanding what the consequences of elevated levels of LXR expression in AS is now a matter of paramount importance, representing an entirely novel and unexpected outcome from the present studies.

12 References

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13 Publications